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THE MECHANISM OF THE INHIBITION OF HEMOLYSIS

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This paper is concerned with the general nature of the reactions involved in the phenomenon of the inhibition of hemolysis by a variety of substances (serum, hemoglobin, glucose, etc.). Since the investigations of Bayer (1907), who showed that the inhibition produced by serum on bile salt hemolysis is largely accounted for by its protein content, the literature has been dominated by the idea that inhibitors such as the serum proteins produce their effect by reacting with the lysin in the same sort of way as when reactant A precipitates reactant B from solution. More specifically, the inhibition produced by serum, plasma, and hemoglobin has been described by supposing that a quantity Q of the inhibitor "removes" (in the same sense as cholesterol, an inhibitor of digitonin hemolysis, precipitates digitonin quantitatively) a quantity Δ of the lysin, so that the original concentration c_1 of the lysin falls to a concentration c_2 , and where $c_1 - c_2 = \Delta$. This treatment of the problem is unsatisfactory for three reasons. (1) The relations between Q and Δ turn out to be very complex. (2) The inhibitory effects of serum, plasma, and hemoglobin have to be treated in a manner entirely different from that used to treat the inhibitory effect of substances such as the sugars and certain electrolytes. (3) This treatment of inhibitory phenomena is quite different from that used to treat acceleratory phenomena. There is, of course, no *a priori* reason why the inhibition produced by serum and plasma, the inhibition produced by substances such as glucose, and the acceleration produced by substances such as benzene and indol, should all be brought about in the same way; the possibility of a single underlying mechanism, however, ought to be more thoroughly investigated than it has been.

I. The Inhibitory Effect of Serum and Its Protein Content

The inhibitory effect of serum on saponin (or digitonin) hemolysis is not entirely paralleled by its protein content. Two kinds of experiment show this.

1. One takes a number (six or so) of specimens of human plasma with varying protein content, and finds the asymptotes of the time-dilution curves for saponin in the absence and in the presence of the inhibitor plasma diluted 1 in 1000. This is done by preparing a series of saponin concentrations in phosphate buffer (pH = 7.3) varying by steps of 5 γ /cc. upwards from 50 γ /cc. to about 200 γ /cc. One series is put up without the addition of inhibitor, the

systems consisting of 0.8 cc. of lysin, 0.8 cc. of buffer, with 0.4 cc. of red cell suspension added, and the asymptote of the time-dilution curve is found by observing the concentration c_2 which produces complete hemolysis after 300 minutes (for technique, see Ponder, 1934). A second series is set up with 0.8 cc. of plasma in a dilution of 1 in 1000 replacing the 0.8 cc. of buffer, and the asymptotic concentration c_1 for this series is found in a similar way. Table I

TABLE I

Plasma	System	Protein	Asymptotic concentrations c_1 and c_2	Δ	R
		<i>gm. per cent</i>			
1	Standard + plasma 1:1000	—	36	16	1.44
		7.6	52		
2	Standard + plasma 1:1000	—	35	17	1.48
		7.3	52		
3	Standard + plasma 1:1000	—	38	14	1.37
		6.6	52		
4	Standard + plasma 1:1000	—	35	15	1.43
		6.2	50		
5	Standard + plasma 1:1000	—	38	12	1.32
		5.9	50		
6	Standard + plasma 1:1000	—	36	10	1.28
		5.8	46		

shows how the difference $\Delta = c_1 - c_2$ and the ratio $R = c_1/c_2$ vary with the protein content of the plasma. Both the difference Δ and the ratio R are given, because it will be shown directly that these two measures of the extent of the inhibition imply the existence of quite different mechanisms.

2. These determinations of the position of the time-dilution curve asymptotes in the absence and in the presence of the inhibitor are troublesome, partly because of the long times involved, and partly because of the difficulty in reading the 300 minute end-points. One can arrive at the same result by plotting a time-dilution curve for saponin, selecting a concentration c_1 (say 1 in 33,000 in phosphate buffer) which gives complete lysis in a relatively short time (about

5 minutes), and then finding the concentration c_2 which appears to be present when sera in dilution of 1 in 5000 are added to the system. Either $\Delta = c_1 - c_2$, or $R = c_1/c_2$, would be a measure of the inhibition, and what we find is that neither of these functions is wholly determined by the protein content of the serum. This is shown in Table II.

The conclusion to be drawn is that the inhibitory effect on saponin hemolysis is not entirely dependent on the protein content of the plasma. Farley (1942), indeed, has suggested that plasma contains powerful inhibitors other than proteins, and the poor correspondence between inhibitory effect and plasma protein content shows that this possibility has to be recognized. This idea is not altogether new, for it has already been suggested that the effect of plasma on hemolysis, while in general inhibitory, is a statistical effect of a mixture of many inhibitors and accelerators (Ponder and Abels, 1936; Ponder, 1937;

TABLE II

	Plasma			
	1	2	3	4
Protein, gm. per cent.	5.92	6.56	6.80	7.21
Globulin, gm. per cent.	2.46	2.70	2.90	2.90
Albumin, gm. per cent.	3.46	3.86	3.90	4.31
A/G ratio.	1.4	1.4	1.3	1.5
Δ	14	22	16	20
R	1.26	1.46	1.30	1.40

Collier and Allen, 1942). The question then arises as to whether we ought not to treat it in the same way as we treat acceleratory phenomena and as taking place at the cell surface rather than in the lysin-inhibitor phase.

When we write $\Delta = c_1 - c_2$, we imply that the inhibitor reacts with the lysin c_1 , and renders inert a quantity Δ of it; the amount of lysin which remains is then c_2 . When we write $R = c_1/c_2$, quite a different mechanism is implied. In a system containing sucrose (an inhibitor), for example, a concentration of lysin c_1 produces lysis as if it were of the smaller concentration c_1/R ($R > 1.0$) acting on the cells in the absence of sucrose. This, of course, is the same thing as saying that the resistance of the cells is increased R times by the sucrose, R times more lysin being needed to combine with the membrane component in the presence of the sucrose to produce the same effect as in its absence. The reaction implied by expressing the inhibition as a value of Δ , a function of c_1 , or of c_2 , is one in which the inhibitor reacts with the lysin, but in which the locality of the reaction is not specified (although it has been usually considered as being in the bulk of the fluid of the hemolytic system); when the inhibition is expressed as a value of R , however, the site of the reaction is specified

as being at the cell surfaces, and the effect of the inhibitor has to be regarded as in general similar to the effect of an accelerator (for which see Ponder, 1941). The description of inhibition in terms of R values has, indeed, been used in the case of inhibitors such as certain sugars and salts ever since the R notation was introduced (Ponder, 1926; and see Kennedy, 1926; Bodansky, 1928; Yeager, 1929; and Gordon, 1933 *a* and *b*), while, except in one instance (Kennedy, 1925), Δ has been used to describe the inhibition only in the cases of the inhibitors plasma, serum, and hemoglobin (Ponder, 1924, 1925, 1932; Ponder and Gordon, 1934).

From a mathematical point of view, the expressions $\Delta = c_1 - c_2$ and $R = c_1/c_2$ transform into one another, and are two different ways of expressing the same experimental results. The relation between the two expressions has been fully discussed already (Ponder, 1934, pp. 200-205). In considering the inhibition produced by plasma and serum in terms of R instead of Δ , we can accordingly retain all the existing experimental results, although we may change our interpretation of them. It is first necessary, however, to show that a change in interpretation is necessary, and so I shall next consider some hitherto undescribed properties of saponin-plasma-cell systems.

II. Certain Properties of Saponin-Plasma-Cell Systems

1. *The Temperature Coefficient.*—In 1925 I was unable to find any clear cut temperature dependence for the inhibition of serum on saponin hemolysis (Ponder, 1925) although there were indications that the inhibition becomes less as the temperature increases. The failure of these experiments to show a definite effect of temperature was probably due to the method used for analyzing the curves (in terms of the slope of $\log c$ plotted against $\log \Delta$). Much more satisfactory results are obtained when time-dilution curves for standard systems and for saponin-inhibitor-cell systems are plotted and analyzed in terms of R values. Table III shows the average values of R obtained at two different temperatures for systems containing saponin, human red cells, and (*a*) plasma with a protein content of 7.0 gm. per cent, and (*b*) M/3.5 sucrose.¹

In each case the values of R show that the temperature coefficient is small but negative, and therefore the same sign as the temperature coefficient for accelerators (Ponder, 1941). This result can be interpreted as pointing to surface phenomena being involved in the reaction which brings about inhibition, just as it has been shown to be in the case of acceleration (Ponder, 1939). In the case of inhibition by plasma, the surface might be either the extended

¹ Since R is a function of c_1 , the temperatures chosen should be such that the same range of lysin concentration is covered by the time-dilution curves for the standard systems. This requirement considerably limits the range of temperatures over which R values can be properly compared. The standard curves can be plotted over the same concentration range at 22°C. and at 37°C., for example, but not at 22° and at 10°.

surface of the colloidal inhibitor or that of the red cell; in the case of inhibition by sucrose, on the other hand, the latter is almost certainly involved.²

2. *The Inhibition in Systems Containing Different Kinds of Cell.*—The extent of the inhibition of saponin or digitonin hemolysis produced by plasma or serum varies according to the type of red cell used in the hemolytic system. Table IV shows the values of R obtained in systems containing 0.8 cc. of saponin in dilutions from 1 in 10,000 to 1 in 35,000 (22°C.), 0.8 cc. of plasma diluted 1 in 500, and 0.4 cc. of a standard suspension of the cells of the sheep, man, rat, and guinea pig.

For any quantity of lysin c_1 , the R values are in the order sheep > man > rat > guinea pig, so the extent of the inhibition depends on the type of cell used

TABLE III

Inhibitor	Quantity	R , 22°C.	R , 37°C.
Plasma.....	0.8 cc. of 1 in 250	1.23	1.14
Sucrose.....	0.8 cc. of 0.286 M	1.21	1.12

TABLE IV

1 in	Sheep	Man	Rat	Guinea pig
10,000	1.80	1.23	1.20	1.13
15,000	1.67	1.19	1.14	1.11
20,000	1.60	1.15	1.11	1.10
25,000	1.50	1.15	1.10	1.08
30,000	1.40	1.13	1.08	1.07
35,000	1.31	1.10	1.06	1.06

in the hemolytic system. It is virtually impossible to reconcile this result with the idea that the inhibitor reacts solely with the lysin in the bulk of the system, removing an amount Δ by forming an inactive compound with it. The inhibition is clearly the result of some reaction which involves the cell surface, and which varies with its nature.

3. *R As a Function of c_1 .*—Inspection of Tables IV and V will show that the value of R is not constant in systems containing either plasma or sucrose as inhibitors, and that R falls steadily as c_1 decreases, so that the curves relating c_1 and R are convex to the R axis (Fig. 1).

In the case of the inhibition produced by most sugars and certain electrolytes, it has been recognized at least since 1928 that R falls with c_1 , Ponder and Yeager

² Even in the case of a substance such as sucrose, the possibility of a surface reaction in the bulk of the system has to be considered, for Kleinberg (1933) has shown that saponin may exist in a micellar state.

(1928) giving values of R which decrease from 1.50 to 1.25 for taurocholate-sucrose-human cell systems. In some systems, however, the curve relating

TABLE V

1 in	c_1	R human cells and sucrose	R sheep cells and plasma
10,000	200	1.40	1.80
15,000	133	1.31	1.67
20,000	100	1.25	1.60
25,000	80	1.20	1.50
30,000	67	1.13	1.40
35,000	57	1.09	1.31
40,000	50	1.07	1.25

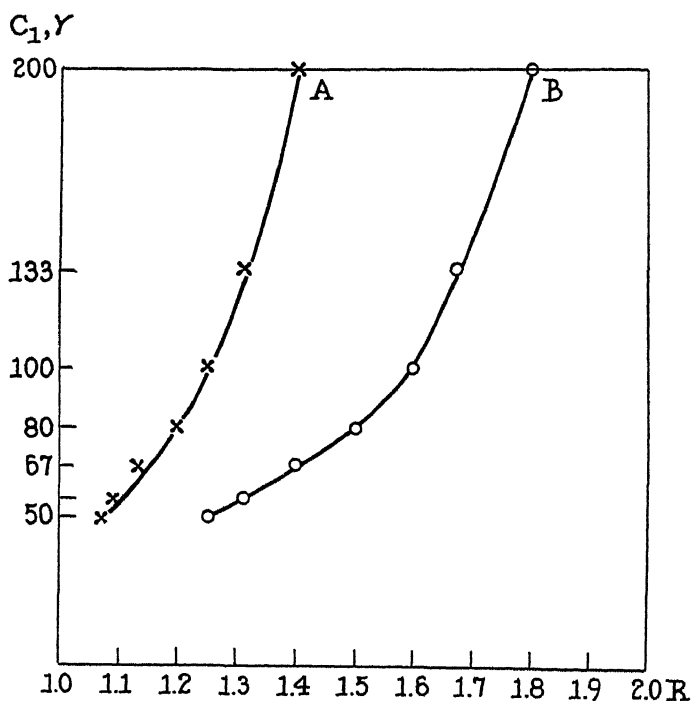


FIG. 1. R as a function of c_1 . Curve A, inhibition of saponin hemolysis (human cells) by $M/3.5$ sucrose. Curve B, inhibition of saponin hemolysis (sheep cells) by plasma.

R and c_1 can be approximately represented by a straight line within a limited range of lysin concentration. Because it is only an approximation to the curve, this line usually makes a small intercept on one of the axes. In such cases, R can be treated as substantially constant, as in Yeager's investigation of the in-

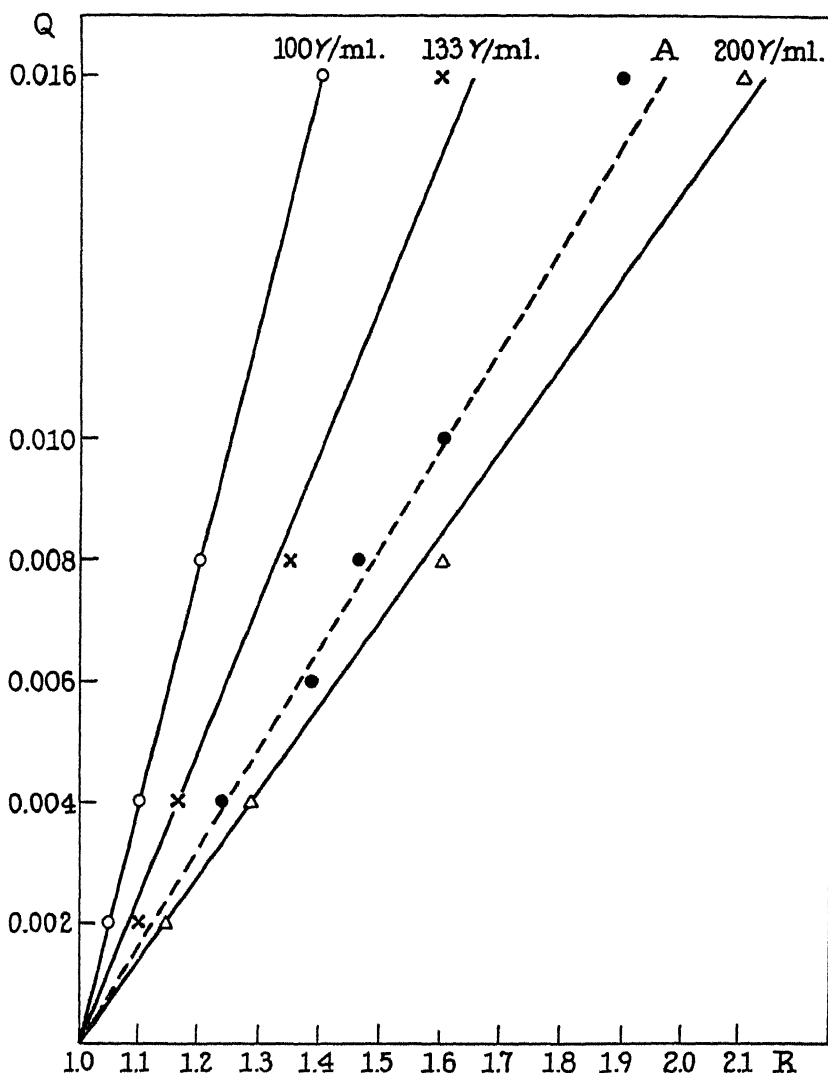


FIG. 2. R as a function of Q . Inhibition of saponin hemolysis (human cells) by plasma in various amounts Q ; data for three initial concentrations of lysin, from 100 γ /ml. to 200 γ /ml. Curve marked A: data from Ponder and Gordon (1934), included in this figure only for the purpose of illustrating linearity. The absolute values of Q shown on the ordinate do not apply to Curve A.

hibitory effect of sucrose (1929) but where the relation is too curvilinear for the fall of R with c_1 to be ignored, the expedient has been resorted to of measuring the extent of the inhibition by the ratio of the asymptotic values of c_∞ for the

standard system and for the inhibited system respectively; *i.e.*, $R = c_{1\infty}/c_{2\infty}$. Yeager (1928), in his paper on the Rywosch series with taurocholate, and Gordon (1933 *b*), in his investigation of the effects of electrolytes, have used this method of expressing inhibitory effects, and so have avoided the difficulties presented by the R, c_1 relation being curvilinear; evidence of its being so, however, appears in all the papers on the subject of inhibition of hemolysis up to the present time.

In the case of the inhibition produced by plasma, serum, and hemoglobin, a relation between Δ and c_2 (Ponder, 1924, 1925), or between Δ and c_1 (Ponder and Gordon, 1934) has been used instead of a relation between R and c_1 principally because the R, c_1 relation is so obviously curvilinear as to apparently separate it from the nearly linear relation met with in the case of the inhibition produced by many sugars, electrolytes, etc. Looking back on the development of the subject, it is now clear that the R, c_1 relation is *always* curvilinear if the experimental range is sufficiently extended, and that the Δ, c_1 relation, which first attracted attention because of the idea that the inhibitor forms an inactive compound with the lysin in the bulk of the fluid in the system, represents an attempt to distinguish between two kinds of hemolytic system (sugar-lysin-cell systems and plasma-lysin-cell systems) between which there is no real difference.

4. *R As a Function of Q.*—It has been known since 1924 that the inhibition produced by a quantity of serum Q increases as Q increases. Although a relation between Δ and Q , which turns out to be curvilinear and complex, has been obtained (Ponder, 1924, 1925; Ponder and Gordon, 1934), no one seems to have considered R as a function of Q . The best existing values are those of Ponder and Gordon (1934, their Table II), and plotting R against Q gives the line marked A in Fig. 2. The relation is a linear one over a very extended experimental range, and the slope of the line depends on the concentration of lysin in the system, since R is also a function of c_1 . The other lines in Fig. 2 illustrate this.³

DISCUSSION

The first point which emerges from these experiments is the similarity between inhibitory and acceleratory phenomena. Both appear to involve the region of the surface of the red cell (*cf.* Ponder, 1939, 1941), both have a negative temperature coefficient, in both cases R , the measure of the amount of

³ In the case of inhibition of sodium taurocholate hemolysis by plasma, the R, Q relation is better represented by a curve convex to the R axis than by a straight line (Ponder and Gordon, 1935). It is possible, indeed, that the relation would be curvilinear even in the case of saponin if the experimental range were to be sufficiently extended.

inhibition or acceleration, is an approximately linear function of the quantity of inhibitor or accelerator Q , and in both cases R is a function of c_1 .⁴

It should be observed that these relations apply to all inhibitors yet studied, just as they apply to all accelerators, and since it has been shown (Ponder, 1926; Ponder and Yeager, 1928) that inhibitors such as sucrose react more or less irreversibly with components of the cell membrane just as accelerators do, we have to entertain the hypothesis that the inhibitors contained in plasma react with the components of the red cell membrane so as to increase their resistance to the action of hemolysins.

This idea is not so strange as might appear at first sight, quite apart from the fact that plasma apparently contains inhibitors other than the plasma proteins (Section I, above), and that some of these inhibitors may be substances of low molecular weight which can be imagined to react with membrane components. When considering the inhibitory effect of the plasma proteins, it should be remembered that some of the protein components of the membrane can leave the cell surface to appear in the surrounding fluid, while some of the protein components of plasma can leave the fluid to take up positions in the membrane and apparently to become part of its structure. This transferability is shown in the clearest way in the experiments of Furchgott (1940) and of Furchgott and Ponder (1940) on the disk-sphere transformation between glass slide and cover glass. The "anti-sphering substance," identified as a crystalbumin, leaves the cell surfaces to be adsorbed on the glass, and at the same time the cell becomes spherical. The lost anti-sphering substance can be replaced in the cell surface by adding plasma, which reconverts the spheres into disks. A 30 per cent change in surface area is involved in these transformations, and the amount of crystalbumin which leaves and returns is sufficient to form about one-third of the estimated mass of the membrane, although it would form a layer only a few molecules thick if spread over the cell surface. A protein can accordingly be thought of as reacting with components of the membrane ultrastructure.

The process underlying the phenomena of lysis and its acceleration or inhibition accordingly seems to be one in which the lysin reacts with a component

⁴ The R, c_1 curve in the case of an accelerator can be analyzed (Ponder, 1941), a sharp curvature occurring at a point corresponding to the asymptote of the standard curve. R is virtually constant below this point and linear with c_1 below it. The form of the R, c_1 curve in the case of the inhibitors is as yet unaccounted for. Since the asymptote for the inhibited system corresponds to a greater value of c_1 than that for the standard system, an analysis parallel to that for accelerated systems is not possible, but the underlying explanation is probably fundamentally similar, *viz.* the inhibitor is present at the cell surface in excess, its effect increasing as the concentration of lysin present at the surface, and therefore capable of being inhibited, increases.

or components of the cell membrane in such a way as to break down its semipermeability to hemoglobin, and in which the accelerator or inhibitor also reacts with the component in such a way as to increase or decrease the effectiveness of the lysis in producing breakdown. By breakdown we mean the local or general dissolution of the continuity of the membrane components upon which the properties of semipermeability depend, and we express the change in effectiveness by saying that R times as much lysis is required in the presence of the accelerator or inhibitor in order to produce the same destructive effect, or that the accelerator or inhibitor changes the resistance of the membrane to the lysis by a factor R . This statement is sufficient so far as an investigation into the kinetics of lysis and its acceleration or inhibition is concerned, but before it can have any real meaning in terms of mechanism it is necessary both to identify the components which enter into the reactions and to show how the reactions result in hemolysis, accelerated or inhibited. The relations between quantities, expressed by the equations for the kinetics, must correspond to actual occurrences on the molecular scale, and if the reactions give a few hints as to the nature of the occurrences, we cannot omit following them up. There are several points which are of particular interest in this connection.

1. The conclusion has already been reached that lysis occurs through a breakdown of spots or patches in the cell membrane, although the lysis acts over the surface of the membrane as a whole, these spots ("key spots") being regions at which the resistance to the lysis is particularly low (Ponder, 1941). The combined area of the spots can be inferred from experiments on the "fading time" or length of time taken for the hemoglobin to diffuse out of a single cell during its hemolysis (Ponder and Marsland, 1935). In systems in which approximately asymptotic concentrations of lysis are present, the fading time is about 15 seconds, and this corresponds to a value of Nd of 0.25, where N is the number of holes or permeable patches, and d their mean diameter in μ . Considering that hemoglobin diffuses through these holes, and that the diameter of the hemoglobin molecule is in the neighborhood of 50 Å, the minimum mean diameter of the holes would be 50 Å, and in an approximately asymptotic concentration of lysis for which $Nd = 0.25$, the maximum number N would be 50. These observations show that the structure of the membrane is discontinuous, certain spatially separated areas breaking down more readily than others as a result of the reaction with the lysis.

2. If the resistance of the least resistant spots is x_0 , there must also be present a number of spots of greater resistance, x_1 , x_2 , and so on. If N is the number of spots of any degree of resistance as measured in terms of x , the plotting of N as a function of x will give rise to some kind of frequency distribution, the integral of which is the experimental curve obtained by plotting the average fading time of the cells against the concentration of lysis in the system (Ponder and Marsland, 1935; Ponder, 1941). The distribution is very skew, which

means that a small part of the surface is made up of areas which are relatively readily broken down as lysin is transformed, while the greater part is made up of areas which have a higher and much less uniform resistance.

3. The breakdown of the spots of resistance x_0 , x_1 , etc., is not the first change which occurs in the red cell membrane under the action of the hemolysin, for lysis is always preceded by a loss of the discoidal form. The membrane ultrastructure must therefore (a) preserve a surface greater than the minimum for the enclosed volume, thus maintaining the discoidal form, (b) undergo some modification as a result of the action of the lysin, so that the rigidity of form is lost, and the expanded surface cannot be maintained, and (c) break down at spots as a result of the further action of the lysin. Like the change which results in loss of semipermeability, the form change is a process which has a measurable velocity. It does not occur immediately on the addition of the lysin, but only after a "latent period;" the first signs of it then appear at localized areas on the surface, so that the discoidal form develops crenations in increasing number as the disk passes into the sphere. Just as the membrane ultrastructure has spots of varying resistance to permeability breakdown, so it seems that it has spots of varying rigidity of form; in the case of any one cell, however, the shape change is always complete before the least resistant key spot breaks down.

4. The spots of resistance x_0 can be thought of as being distributed through the membrane ultrastructure either at random or in some kind of pattern, but since the number of these spots may be as small as 50, the unit pattern must be large enough to cover some 10^8 \AA^2 before repeating itself. This is a very large unit pattern. The same alternative applies to the spots of greater resistance and to the spots of varying rigidity of form. While there is not conclusive evidence in favour of it, the idea of a pattern is the more attractive of the two, if for no other reason than that the cell has a regular geometrical form.⁵

⁵ Wrinch (1942) has put forward an attractive hypothesis regarding the general nature of the membrane ultrastructure. In her model the skeleton of the membrane is composed of multiply connected proteins arranged in a definite pattern. Because of differences in their side chains, these protein molecules and their linkages are not all the same, and this would permit of local differences in reactivity with lysin molecules. The size of a protein unit in the model is from 50 to 100 \AA , which roughly corresponds to the size of a key spot, and the model would not exclude a sufficiently infrequent repetition of the unit pattern to allow for the number of spots of resistance x_0 being comparatively few. Cephalin molecules are conceived of as being attached to the protein network with a more or less normal orientation to it, and the network may be not single, but multiple; in this way a laminated structure may be built up, and the polarization optics of such a structure would be something of the kind already shown to exist for the membrane of the red cell ghost (Schmitt, Bear, and Ponder, 1936, 1938). From the standpoint of the red cell, Wrinch's model is interesting not only because it pictures the membrane as having a molecular pattern, but because it

5. The fact that the same value of R is obtained by comparing a system containing an accelerator or an inhibitor with a standard system, irrespective of the percentage of hemolysis used as an end-point (Ponder, 1934, p. 190), shows that the effect of the accelerator or inhibitor is to change the resistance of every reactive spot in the membrane ultrastructure by a factor R . The resistance of each area is accordingly changed by a constant factor and not by a constant amount. This result suggests that the accelerator or inhibitor has some over-all effect, the simplest example of which would be that of changing the extent to which the lysin is concentrated at the surface, or is partitioned between the material of the membrane and the surrounding fluid. Some kind of combination between the accelerator or inhibitor and the membrane ultrastructure is presumably involved. Part of the effect of most accelerators and inhibitors is reversible by such a simple procedure as washing (Ponder, 1926, 1939), and this probably corresponds to a loose adsorption-like combination involving large parts or the whole of the surface. Some of the loose links are later replaced by more permanent combinations involving the same types of bond as are broken down by lysins; the accelerator or inhibitor so combined cannot be reversed by washing, and many accelerators *and inhibitors* (e.g. sucrose) themselves produce lysis if present in sufficient concentration.

SUMMARY

The principal conclusion of this investigation is that the inhibitory effect of plasma or serum on hemolysis by saponin and lysins of the same type is similar in nature to the inhibitory effects of certain sugars and electrolytes, which again are similar to the acceleratory effects produced by indol, benzene, and other substances already studied. All these effects, both inhibitory and acceleratory, are the result of reactions between the inhibitors or accelerators and those components of the red cell membrane which are broken down by lysins.

The inhibitory effect of plasma on saponin hemolysis has a number of properties in common with the inhibition produced by sugars and electrolytes and with accelerations in general. (a) The temperature coefficient is small and negative. (b) The extent of the inhibition depends on the type of red cell used in the hemolytic system. (c) The most satisfactory measure of the extent of the inhibition, the constant R , is a function of the concentration of lysin in the system, and (d) R is a linear function of the quantity of inhibitor present.

supposes two sets of structures, the first of which tends to form the smallest surface for the enclosed volume and the second of which may, under the proper circumstances, tend to form an expanded surface. This is reminiscent of Norris' theory (Norris, 1882; Ponder, 1933), with the distinction that the expansive component in Wrinch's model would be the protein network, while in Norris' theory it is the layer of lipoid molecules.

It is also shown that the inhibitory effect of plasma and serum is not entirely dependent on its protein content.

The process underlying the phenomenon of lysis and its acceleration or inhibition seems to be one in which the lysin reacts with a component or components of the cell membrane in such a way as to break down its semipermeability to hemoglobin, and in which the accelerator or inhibitor also reacts with the same component in such a way as to increase or decrease the effectiveness of the lysin in producing breakdown. The membrane is considered as being an ultrastructure made up of small areas or spots of varying degrees of resistance to breakdown, the resistances being distributed according to a negatively skew type of frequency curve, and the process of lysis seems to begin with the least resistant spots breaking down first. These spots may be arranged in some regular spatial pattern, and the membrane has also to be regarded as possessing spots of varying rigidity of form. The accelerator or inhibitor changes the resistance of every reactive spot in the ultrastructure by a factor R , which suggests that acceleration and inhibition are results of some over-all effect, such as that of changing the extent to which lysin is concentrated at the surface or partitioned between the material of the membrane and the surrounding fluid. Some kind of combination between the accelerator or inhibitor and the material of the ultrastructure is presumably involved; at first the combination seems to be a loose one and partly reversible, but later some of the loose links are replaced by more permanent combinations involving the same types of bond as are broken down by the lysins themselves.

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AN EXPERIMENTAL SEPARATION OF OXYGEN LIBERATION FROM CARBON DIOXIDE FIXATION IN PHOTOSYNTHESIS BY CHLORELLA*

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In the process of photosynthesis in *Chlorella* it is now known that the oxygen liberated during CO₂ fixation and reduction originates from water (Ruben *et al.*, 1941). It is further known that in some algae (not *Chlorella*) one can obtain carbon dioxide fixation and reduction without the release of oxygen (by adaptation to hydrogen, Gaffron, 1940, 1942 *a*, 1942 *b*, Gaffron and Rubin, 1942) and that in isolated chloroplasts one can obtain oxygen (by the reduction of ferric salts, Hill, 1940, Hill and Scarisbrick, 1940 *a*, 1940 *b*) without a simultaneous reduction of carbon dioxide. It therefore appears that, since the reactions of CO₂ fixation and O₂ liberation are only "loosely connected" (Franck and Gaffron, 1941), it should be possible by the choice of an appropriate technique, to cause oxygen liberation in intact cells without the simultaneous reduction of carbon dioxide; *i.e.*, to separate the reactions liberating oxygen from those concerned with carbon dioxide fixation. The results reported in this paper are based upon the concept that if such is indeed the case, it should be possible to obtain oxygen from illuminated *Chlorella* cells by supplying reducible materials other than carbon dioxide.

Preliminary Studies

The technique we have employed is illustrated by the data given in Fig. 1. The algal strain used was *Chlorella pyrenoidosa*, previously described, grown in the light with CO₂ as the sole carbon source in the medium and under the same conditions as previously recorded (Manning *et al.*, 1938). 1 ml. of a washed suspension (washed and suspended in saline) obtained after 10 days growth and containing 96 mg. dry weight of algal cells per ml. was placed in each of three Warburg flasks. 1 ml. M/30 phosphate buffer (pH 7.0) was added, 0.2 ml. 20 per cent KOH placed in the center well, and 0.5 ml. of water, 0.5 ml. of M/10 acetaldehyde, or 0.5 ml. M/10 of benzaldehyde was supplied to the side arms. These were placed in the water bath at 28°C.

Oxygen uptake was determined in the dark at 5 minute intervals for 20 minutes. The shaking rate employed throughout was 115 two cm. strokes per minute; well above that necessary for instantaneous gas exchange. The flasks were then illu-

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minated with approximately 900 foot candles of light (determined with a Weston photometer) obtained from two photoflood bulbs suspended 1 foot above the bath. After illumination of the flasks containing water or acetaldehyde in the side arm (Fig. 1) there is a release of gas for the first 10 minutes (20 to 30 minutes) after which there is a slow uptake. This gas is presumed to be oxygen from traces of CO₂ in solution, from the reduction of intermediates between CO₂ and the final product of photosynthesis, or perhaps resulting from the "CO₂ gush" reactions described by Blinks and Skow (1938) and Emerson and Lewis (1941). As shown in Fig. 1, this gas release tended to vary from flask to flask. However in a relatively short time

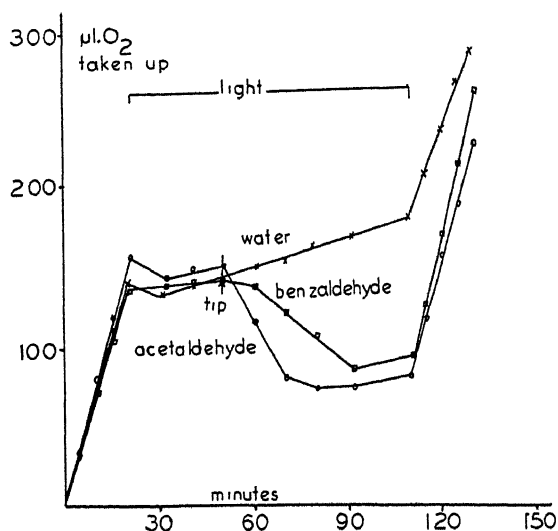


FIG. 1. The production of oxygen from acetaldehyde and benzaldehyde. 1 ml. algal cell suspension (96 mg. dry weight), 1 ml. M/30 phosphate buffer, pH 7.0; 0.2 ml. 20 per cent KOH in center well. 0.5 ml. of acetaldehyde (M/10), benzaldehyde (M/10) or water in side arm. 28°C. Illumination, 900 f. c.

(10 minutes or less) the curves became constant. Illumination was continued. Since the light intensity was above the compensation point this latter period served to remove traces of carbon dioxide in the algal suspension, which had not been completely removed by absorption in the KOH present. The side arm, containing the agent to be studied, was then tipped in and illumination continued for a period. The addition of water caused no change in the oxygen uptake observed in the light. The addition of acetaldehyde or benzaldehyde caused the production of a gas. This gas was not CO₂ (since the KOH would have reabsorbed it). If benzaldehyde is added to an illuminated algal suspension in an atmosphere of nitrogen (under conditions comparable to those of Fig. 1), a gas is evolved. If replicate flasks containing either alkaline pyrogallol or yellow phosphorus in the side arm are employed, no pressure change is found. We therefore conclude that the gas evolved in the first instance is oxygen since it appears to be absorbed by alkaline pyrogallol or yellow

phosphorus. Also, the gas evolved can be detected and measured by the dropping mercury electrode used under the conditions described by Petering *et al.* (1939) and since this is specific for oxygen (under the conditions employed) there seems to be no need to include data on this point here.

Using this method (as in Fig. 1) we were able to obtain appreciable quantities of oxygen (50–100 μ l.) from ferric phosphate and other ferric salts, acetaldehyde, benzaldehyde, parabanic acid, nitro-urea, and sodium carbonate (the latter being a source of CO_2). We were unable to obtain oxygen from the following materials: potassium nitrate, potassium dichromate, *p*-dimethylaminobenzaldehyde, formaldehyde, butylaldehyde, dimethylglyoxime, cystine, alizarin, quinalizarin, methylene blue, urea, methyl urea, cyanuric acid, allantoin, uracil, xanthine, alloxan, succinate, citrate, fumarate, acetate, lactate, malate, isocitrate, pyruvate, glucose, xylose, arabinose, hexose diphosphate, hexose monophosphate, or phosphogluconic acid. It is of some interest that while parabanic acid and nitro-urea were able to cause oxygen liberation, urea and methyl urea were not. This is probably a reflection of the ring structure of crystalline urea (Werner, 1923) and of the fact that the methyl group of methyl urea is attached to the oxygen rather than to the nitrogens. Parabanic acid and nitro-urea both have free carbonyl groups. It is also evident that the reactions with which we are dealing are not the result of a simple reducing action of illuminated tissue since only a relatively small number of materials are capable of causing oxygen production and a slight change in their structure alters this reaction.

With intact *Chlorella* cells we were unable to use the technique of Hill and Scarisbrick (1940 *b*) (whereby the ferrous salt formed during the reduction is prevented from rapid reoxidation in the cells by combination with ferricyanide) in order to study the quantitative relationships between the oxygen produced and the ferric salts supplied. The ferricyanide is itself reduced by the algal suspension in light and the resulting complex of reactions so far defies precise analysis. We therefore chose benzaldehyde as an agent for the study of the oxygen-liberating process since it seemed to act in much the same manner as acetaldehyde but was not as volatile. It could also probably be excluded as either a normal intermediate in photosynthesis or a convenient source of carbon dioxide.

Quantitative Relationships between Benzaldehyde Supplied and Oxygen Liberated

The amount of oxygen liberated from a given quantity of benzaldehyde was found to depend upon three primary factors: (1) the length of time the benzaldehyde had been in contact with the algal suspension before the illumination period; (2) the length of the illumination period before the addition of benzaldehyde; (3) the "physiological state" of the algal suspension. (Three "states" could be distinguished as described below.)

The first two factors are illustrated in Fig. 2. A series of replicate flasks were prepared with each containing 1 ml. washed algal suspension (45 mg. dry weight), 1 ml.

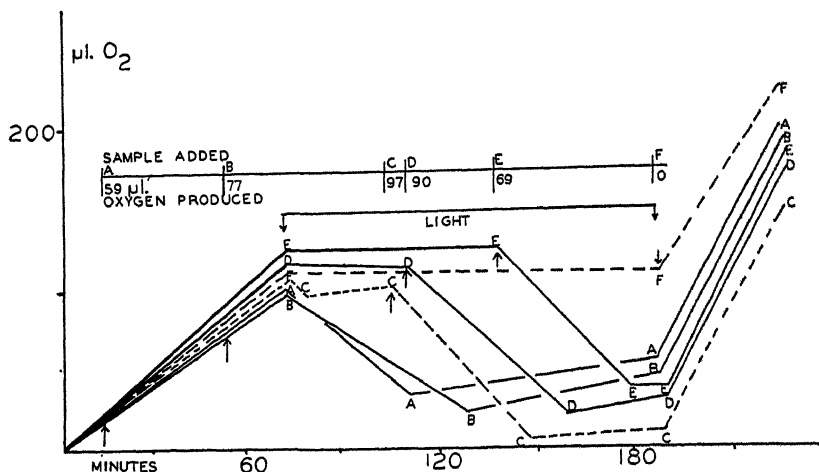


FIG. 2. The influence of the dark period on oxygen production from benzaldehyde. Each flask contains 1 ml. washed algal cell suspension (45 mg. dry weight), 1 ml. $\text{M}/20$ phosphate buffer, pH 7.0; 0.2 ml. 20 per cent KOH in center well. Side arms contained 0.5 ml. of $\text{M}/50$ benzaldehyde which was tipped as indicated. The figures below the line refer to the microliters of O₂ liberated. 28°C. Illumination, 600 f. c. Experimentally determined points have been omitted to avoid confusion. These were determined at 10 minute intervals and connected to produce the lines shown.

TABLE I
Oxygen Production from Benzaldehyde

Flask	Benzaldehyde additions			O ₂ produced μl.
	Time from start	Time in dark	Time illuminated before addition	
	min.	min.	min.	
A	10	60	0	58.9
B	40	30	0	77.5
C	100	0	30	96.8
D	110	0	40	90.2
E	130	0	60	69.4
F	190	0	120	0

$\text{M}/20$ phosphate buffer (pH 7.0), 0.2 ml. 20 per cent KOH in the center well, and 0.5 ml. $\text{M}/50$ benzaldehyde in the side arm. At 10 minutes the benzaldehyde was added in flask A, at 40 minutes the benzaldehyde was added in flask B, at 70 minutes the entire series was illuminated, at 100 minutes flask C was tipped, etc., as indicated in the figure. The amount of oxygen produced in each case is given in Table I. It may be

first noted that with replicate aliquots of the same algal suspension it is possible to alter the amount of oxygen obtained from a given quantity of benzaldehyde. First, less oxygen is produced on illumination the longer the time benzaldehyde has been in contact with the algal cells in the dark; e.g. $Q_{\text{benzald.}}$ in flask A = 1.9, B = 2.2. Second, when benzaldehyde is added in the light the oxygen produced is dependent upon the length of the previous period of illumination; the longer that period, the less oxygen is produced (cf. flasks C, D, E). No oxygen is produced if the benzaldehyde is added in the dark after a long period of illumination (cf. flask F).

Turning to the first effect, it is evident that the decreased oxygen production is not due to the oxidation of benzaldehyde since there is no alteration in the rate of oxygen uptake upon its addition in the dark (cf. flasks A and B). Yet the benzaldehyde has been apparently removed in the dark. A reaction fulfilling such conditions would be a dismutation of the benzaldehyde into benzoic acid and benzyl alcohol. We therefore have studied the possibility of a dismutation of the benzaldehyde as expressed by equation 1.



That such a reaction exists in *Chlorella* is evident from Fig. 3. If benzaldehyde be added in the dark to algal cells suspended in bicarbonate, an "extra carbon dioxide" production due to the formation of acid can be observed (Fig. 3B). This cannot be due to the production of carbon dioxide from the benzaldehyde itself since the latter added in the absence of bicarbonate causes no carbon dioxide formation (cf. Fig. 3A). In neither case does the addition of benzaldehyde alter the rate of oxygen uptake, although these rates are not identical in the phosphate or bicarbonate buffers. This, of course, is a reflection of the influence of ionic balance upon the rate of respiration of *Chlorella*. Genevois (1927) has reported that *Chlorella* respire on acetaldehyde with an R.Q. of 1.3; i.e., excessive carbon dioxide is produced from the acetaldehyde. One can obtain data entirely comparable to those of Fig. 3 using acetaldehyde in place of benzaldehyde. Since Genevois employed bicarbonate buffers throughout his work we feel that the acid production of this type of dismutation was the source of his "extra" carbon dioxide. We have, however, been unable to confirm his observations of a marked increase in oxygen uptake due to the addition of acetaldehyde.

It is evident from Fig. 3B that the amount of carbon dioxide released from the bicarbonate is not stoichiometrically related to the benzaldehyde supplied; i.e., the benzoic acid found is less than that expected from the amount of benzaldehyde added. There must, therefore, be other reactions in addition to the dismutation which remove the benzaldehyde.

The conditions imposed, i.e. that benzaldehyde does not cause an increased respiration when added in the dark, rather severely limit the other types of reactions which can occur. One which has seemed to us probable arises from

an explanation of the second factor observed; *i.e.*, that the amount of oxygen found is dependent upon the length of the previous illumination period. It seemed probable to us that illumination in the absence of carbon dioxide

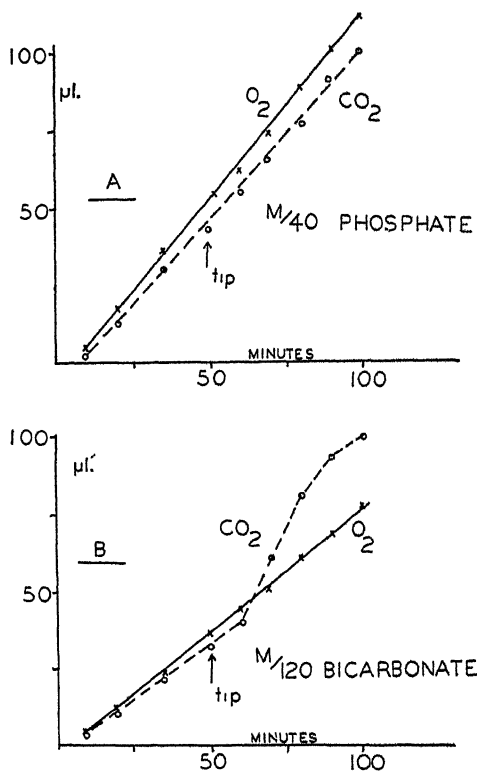


FIG. 3. The production of acid upon the addition of benzaldehyde to *Chlorella* suspensions in the dark.

1 ml. algal cell suspension (0.2 ml. of packed wet cells) (in saline), 0.5 ml. M/50 benzaldehyde in the side arm of each flask. Part A, 1 ml. M/20 phosphate buffer, pH 7.0 initially. One flask with 0.2 ml. 20 per cent KOH for oxygen uptake. Second flask without KOH but supplied with an atmosphere of air plus 5 per cent CO₂ to determine CO₂ production. Part B, as A, with 1 ml. M/60 NaHCO₃ substituted for the phosphate buffer. 28°C. Dark throughout.

would tend to build up reducing materials in the algal cells (and liberate some oxygen). These reducible materials might be sufficiently stable and sufficiently active to reduce some of the benzaldehyde to benzyl alcohol. This would account for the effects observed in Figs. 2 and 3; but further proof that this type of reaction occurs is not available. We have called it an "internal reduction." However, there is no doubt that reactions exist in *Chlorella* leading to the disappearance of added benzaldehyde without either increased

respiration in the dark or oxygen production in the light. The lack, then, of exact stoichiometric relation between benzaldehyde added and oxygen produced (when it exists, see below) is due to chemical reactions which cause the disappearance of the benzaldehyde from the reacting mixture before it can be used in oxygen production.

The third factor involved, that of the "physiological state" of the algal cells is more difficult to control. Using the same species of alga, cultivated on the same medium under the same conditions and handled throughout in a rather closely similar manner, we have obtained suspensions having three different

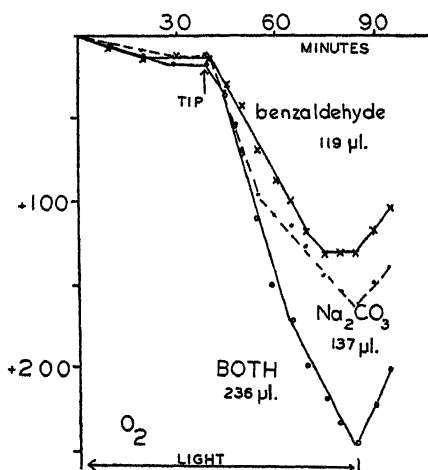
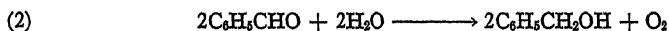


FIG. 4. Oxygen production from benzaldehyde and sodium carbonate. 1 ml. algal cell suspension (0.2 ml. packed wet cells), 1 ml. M/30 phosphate buffer, pH 7; 0.2 ml. KOH in center well of each flask. Benzaldehyde, 0.5 ml. M/50; Na_2CO_3 , 0.5 ml. M/50; mixture, 0.25 ml. M/25 benzaldehyde and 0.25 ml. M/25 Na_2CO_3 . 28°C. Illumination, 600 f. c. over period indicated.

"physiological states." In general, but not invariably, the younger suspensions tend to follow case I while older suspensions tend to follow cases II or III. Suspensions stored in the refrigerator are almost invariably case III. The "physiological states" are described below.

Case I.—In these suspensions all of the benzaldehyde added appears as oxygen in the proportions expressed in equation 2. In these suspensions the



"internal reduction" or the dismutation reactions described above appear to be negligible. Upon the addition of benzaldehyde in the dark in the presence of bicarbonate no appreciable "extra CO_2 " is formed. The action of this type of suspension is illustrated in Fig. 4, which also gives a comparison between the rate of oxygen production from benzaldehyde and from equivalent quanti-

ties of Na₂CO₃ (this will be discussed later in the section on Rates of oxygen production). In this case the addition of 0.5 ml. of M/50 benzaldehyde (224 μ l.) caused the production of 119 μ l. of oxygen (112 μ l. theoretical, *cf.* equation 2). Upon illumination for relatively long periods of time before the addition of benzaldehyde the amount of oxygen produced is less ("internal reduction") and storage of such suspensions in the refrigerator for 1 to 2 days will convert them into Case II or Case III types.

Case II.—In certain other suspensions one is able to account for all of the benzaldehyde disappearing by means of the simultaneous action of reactions expressed in equations 1 and 2.

For example, 224 μ l. of benzaldehyde (0.5 ml. M/50) when added in the light to such a suspension produced 81.6 μ l. of oxygen over a 20 minute period, after which the curve leveled off. When added in the dark in the presence of bicarbonate and 5 per cent CO₂, the same amount of benzaldehyde produced 28.2 μ l. of CO₂ in 20 minutes and continued at that rate for almost 60 minutes. From equation 2, the 81.6 μ l. of oxygen produced would be equivalent to 163.2 μ l. of the benzaldehyde added. From equation 1, the 28.2 μ l. of CO₂ (= 28.2 μ l. of benzoic acid) would be equivalent to 56.4 μ l. of the benzaldehyde added. We have therefore accounted for 219.6 (98 per cent) of the 224 μ l. of benzaldehyde originally added. It is of interest that the oxygen produced is proportional, not to the benzoic acid formed, but to that part of the benzaldehyde which did not form benzoic acid.

We have also found that the dismutation reaction (equation 1) is very sensitive to the presence of iodoacetic acid while the oxygen-liberating reactions are not. In the same experiment as described above, in the presence of M/1800 iodoacetic acid, the addition of 224 μ l. of benzaldehyde caused the production of 114 μ l. of oxygen (equivalent to 228 μ l. of benzaldehyde). All (102 per cent) of the benzaldehyde follows the pathway described by equation 2 in the presence of M/1800 iodoacetate.

Case III.—In still other suspensions (usually older, stored, or excessively illuminated) one is unable to account for all of the benzaldehyde supplied in terms of the reactions expressed in equations 1 and 2. In these cases the addition of iodoacetate (M/600 to M/1200) increases oxygen production about in proportion to that which can be attributed to dismutation but does not enable one to reach stoichiometric quantities. Apparently in these suspensions the benzaldehyde disappears because of the occurrence of both the dismutation and the "internal reduction" reactions.

The conclusion which arises from the experiments outlined above is that the lack of a direct stoichiometric relationship between the oxygen produced and the benzaldehyde added when it occurs is due to the presence of reactions other than that expressed by oxygen production (equation 2) which convert the benzaldehyde into other materials.

Evidence That Carbon Dioxide Is not an Intermediate in the Production of Oxygen from Benzaldehyde

The reaction involving benzaldehyde which is of most interest to us is the one concerned with the production of oxygen (equation 2) rather than the dismutation or the postulated "internal reduction." The fact that oxygen can be produced (sometimes in stoichiometric proportions) when benzaldehyde is added to illuminated *Chlorella* cells may be interpreted in either of two ways: (1) that the benzaldehyde is reduced by the action of light, carbon dioxide playing no part in this reduction, or (2) that the benzaldehyde serves as a source of carbon dioxide and that what really is being observed is a normal photosynthesis (fixation and reduction of carbon dioxide and oxygen liberation from water). It is of interest that the data of Warburg and Negelein (1920) showed that carbon dioxide was an important intermediate in the production of oxygen from nitric acid.

This is a difficult problem to approach particularly in view of the physiological variation encountered, and the apparent complexity of the pathways by which benzaldehyde reacts without producing oxygen. However, it seemed to us that if carbon dioxide were an intermediate in the production of oxygen from benzaldehyde; if somehow, the latter served as a source of carbon dioxide, it ought to be possible to demonstrate the carbon dioxide.

Of course, the addition of benzaldehyde in the dark did not cause the formation of carbon dioxide (Fig. 3 A). Nevertheless, it might do so in the light. If carbon dioxide were produced from the benzaldehyde, and if conditions were so adjusted that carbon dioxide could escape into the air, then, in the presence of KOH some of this carbon dioxide could be absorbed. Hence one could determine whether the benzaldehyde served as a source of carbon dioxide by determining, quantitatively, the amount of oxygen produced from a given quantity of benzaldehyde in the presence or absence of KOH. In order to work in the absence of KOH, however, one would need to employ a rather long preliminary light period to be certain of consuming all of the carbon dioxide in the system by normal photosynthesis before the benzaldehyde was supplied. Moreover, if carbon dioxide is formed from the benzaldehyde in the light, one would expect *less* oxygen to be formed in the flasks which contain KOH. The data of Fig. 5 are the result of a study of this possibility. The point which concerns us here is not how much oxygen was liberated but if there was any difference between the amount of oxygen produced from the same quantity of benzaldehyde in the presence or absence of KOH. Obviously at pH 7 in phosphate buffer (parts A and B) there is no essential difference due to the presence of KOH. In citrate (C) at pH 5.8 there is also no difference attributable to the KOH. In all of these cases oxygen was being produced before the addition of benzaldehyde; more oxygen in the presence than in the absence of KOH. In saline (part D) before the addition of benzaldehyde, this situation is reversed.

The origin of the oxygen so produced is not definitely known but presumably it arises from the reduction of reducible materials within the cell. However, in

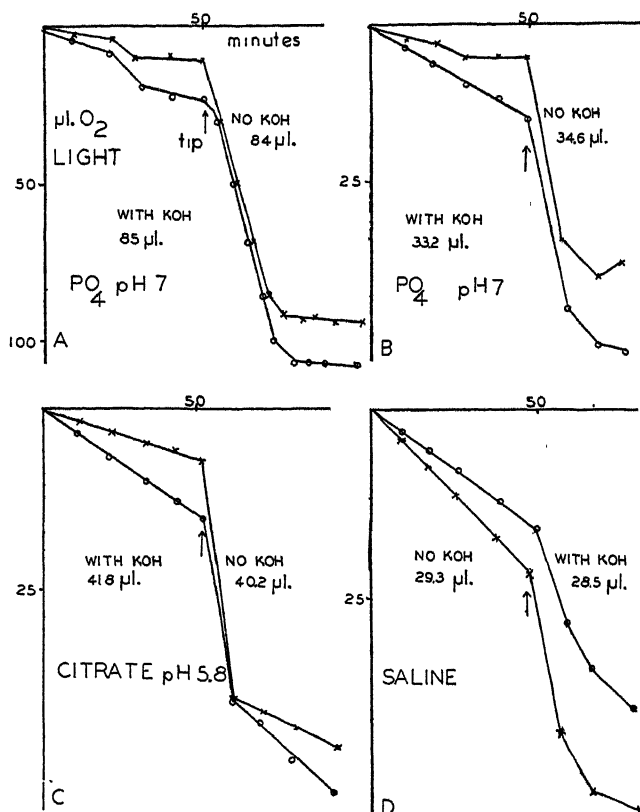


FIG. 5. Oxygen production in the presence and in the absence of KOH. Part A, 1 ml. algal cell suspension (equivalent to 0.06 ml. packed wet cells, suspension 20). 1 ml. M/100 phosphate buffer, pH 7.0, 0.5 ml. M/50 benzaldehyde in side arm; 0.2 ml. 20 per cent KOH in center well. Parts B, C, and D, 1 ml. algal cell suspension (equivalent to 0.06 ml. packed wet cells, suspension 21), 1 ml. 0.6 per cent NaCl, 1 ml. M/100 citrate buffer, pH 5.8, or 1 ml. M/100 phosphate buffer, pH 7.0; otherwise as for Part A. 28°C. Illumination, continued for 90 minutes before the readings recorded in the figure, 600 f. c.

all cases, the addition of benzaldehyde results in no appreciable difference attributable to the KOH.

It might be argued that the previous experiments (Fig. 5) are dependent upon the assumption that the conditions were so adjusted that carbon dioxide, if formed, could escape and that this assumption is not correct. Aside from the fact that at pH 5.5–5.8 the carbon dioxide is all merely in solution and not

in a bound form, and that thus it is in equilibrium with gaseous carbon dioxide (and hence can escape) the data of Table II show that the conditions are such that if carbon dioxide were formed it actually would escape in the sense that added carbonate (whose carbon dioxide pressure is actually zero) has partially escaped into the air at pH 7 (more at pH 5.5) inasmuch as less oxygen is produced from it in the presence than in the absence of KOH (the latter producing the theoretical amount). It should be emphasized that the level of carbon dioxide supplied is such that it is entirely comparable to that presumed to be produced from the benzaldehyde and hence in the latter case there should have been some measurable difference between the presence of KOH and its absence in the form of more oxygen production in the latter case. The only explanation for the results observed (Table II) with the benzaldehyde which is con-

TABLE II
Influence of the Presence of KOH upon the Production of Oxygen from Benzaldehyde and Carbonate

Benzaldehyde			Carbonate		
O ₂ produced from 10 μ M benzaldehyde			O ₂ produced from 10 μ M Na ₂ CO ₃		
pH	+ KOH	- KOH	pH	+ KOH	- KOH
6.8	57.8	60.0	7.0	171	233
5.8	58.5	46.0	5.5	104	223
4.6	49.5	46.0	—	—	—

2 ml. algal cell suspension (0.06 ml. packed wet cells) in M/100 phosphate buffer, pH adjusted with HCl by means of Beckman glass electrode; shaking rate, 115 2-cm. strokes/min. 28°C. Illumination, 600 f. c. for 120 minutes before addition of benzaldehyde or carbonate. Figure recorded is microliters of O₂ produced after addition. KOH when present was 0.2 ml. 10 per cent. Benzaldehyde: suspension 167, theory, 112 μ l. O₂, reaction complete in 20 minutes. Carbonate: suspension 186, theory, 224 μ l. O₂ reaction complete in 15 minutes.

sistent with these facts is that the benzaldehyde did not cause the production of carbon dioxide.

Obviously a stricter test of this technique would be to employ a material which would liberate its carbon dioxide rather slowly. We have, however, been unable to find such a material and attempts to use organic acids (in the hope that they might be decarboxylated) were not successful, in that no oxygen was produced (see preliminary experiments).

We are able to conceive of another possible objection. One could presume that the benzaldehyde liberated carbon dioxide within the cell (only in the light) and that this carbon dioxide did not have time to diffuse out before being used in normal photosynthesis. It is, of course, difficult ever to be sure of the exact nature of reactions which occur in intact cells. One can, therefore, take the position that in this work carbon dioxide has been produced in the inside of the cell (only in the light) upon the addition of benzaldehyde and that its

use in photosynthesis was such that none of it escaped. We, however, have discarded this possibility on the following grounds. First, there is no positive evidence of such internal carbon dioxide production. Second, in view of the extremely rapid transfer of carbon dioxide between the cell and the medium, the concept of an internal carbon dioxide production leads to the idea that the postulated enzymes liberating carbon dioxide from benzaldehyde and those fixing carbon dioxide must be so arranged that the carbon dioxide does not diffuse in the cell from one to the other, since, from purely kinetic considerations, if there were diffusion inside the cell some of the carbon dioxide should escape and hence be detected in experiments such as those recorded in Table II and in Fig. 5. Third, since the benzaldehyde which follows the light reaction (oxygen liberation) does so in accordance with equation 2, this would mean the production of 1 mole of carbon dioxide per 2 moles of benzaldehyde. We consider it exceedingly improbable that *Chlorella* cells are equipped to carry out what must be a rather complex reaction, particularly with a system operating only in the light which also must be closely adjacent to the carbon dioxide fixation system within the cell that the carbon dioxide is not required to diffuse for any distance through the cell interior. Obviously one cannot experimentally approach this problem since so long as the results are negative (*i.e.*, no evidence of carbon dioxide production), it could be argued that the methods were not sufficient to demonstrate the internal production of carbon dioxide. Positive results must be obtained which, so far, have not been observed. But it seems obvious to us that this argument of internal carbon dioxide production from benzaldehyde in the light but not in the dark necessitates such a questionable group of assumptions that it can be discarded on this ground. This situation appears to us to be a justifiable application of Occam's razor and we therefore take the position that these data represent a direct reduction of benzaldehyde and that they do not mean that benzaldehyde serves as a source of carbon dioxide.

It should also be noted that the proof that carbon dioxide is not produced by the addition of benzaldehyde is independent of any assumptions regarding the carbon dioxide content of the solutions at the time of adding the benzaldehyde. We feel that after 10 to 20 minutes' illumination with KOH (and perhaps slightly longer in its absence) carbon dioxide is absent from the solutions. This is, however, a difficult matter to prove since the respiratory system of *Chlorella* in addition to being insensitive to HCN is not sensitive to high concentrations of acid (pH 1-2) and if acid is added in the light in an effort to release any "bound carbon dioxide," an oxygen uptake continues for some time thereafter. There is no gas released upon the addition of acid, but the continuing oxygen uptake obscures small amounts that might be released. It is highly improbable that "bound carbon dioxide" would exist after the treatments employed. The acid, of course, would have very little effect upon dissolved carbon dioxide. We believe the system to be free of carbon dioxide,

since there is no carbon dioxide in the gas phase (KOH present) and since the cells have been illuminated for long periods at light intensities far above the compensation point, which should have served to use up traces of carbon dioxide which had escaped absorption by the KOH.

We are therefore forced to conclude that carbon dioxide is not formed by the addition of benzaldehyde in the light, and that the oxygen formed on such addition results from a more or less direct reduction of the benzaldehyde itself. Since oxygen is here produced, and since carbon dioxide is not the material being reduced, this constitutes a liberation of oxygen without carbon dioxide reduction. This, to our minds, constitutes an experimental separation of the reactions liberating oxygen from those concerned with carbon dioxide fixation.

We are, of course, willing to grant that one or more carrier systems may transport the hydrogen released from water to the reducing enzyme acting on the benzaldehyde. We do not regard benzaldehyde as a "normal" intermediate in photosynthesis (*i.e.*, the production of oxygen by carbon dioxide fixation and reduction) but merely as a rather stable carbonyl group which is able to act with enzymes whose normal substrate is some other compound whose nature can only be surmised.

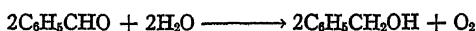
Rate of Oxygen Production from Benzaldehyde

If one examines the rate at which benzaldehyde causes the liberation of oxygen, it will be noted that the highest values obtained approach a Q_{O_2} of +10 whereas it is possible to obtain Q_{O_2} 's for oxygen production from carbon dioxide in excess of +100. This might lead to the position that since the rate of oxygen production was "low" and since there were admittedly unknown dark reactions in operation, it would be hard to concede that a photoreduction of the benzaldehyde indeed occurred. This position, however, results from the neglect of a very fundamental consideration. Q_{O_2} 's greater than +100 occur at relatively high light intensities, and, which is more important, when the cell is bathed in excess carbon dioxide; *i.e.*, when all of its enzyme systems are saturated with carbon dioxide and its products. Actually a comparison of the rates obtained under these conditions with the rates obtained here is not possible; the conditions of operation are entirely different. The only comparisons of value would be between systems saturated with carbon dioxide ($Q_{O_2} = > +100$) and those saturated with benzaldehyde (Q_{O_2} , unknown) or between the rate of oxygen production from Na_2CO_3 (as a source of carbon dioxide) and from benzaldehyde under the same conditions. These latter comparisons (as shown in Fig. 4) show that the benzaldehyde produces oxygen at roughly half the rate of oxygen production from Na_2CO_3 . In this case (Fig. 4) over a period of 15 minutes (maximum rate for carbonate) the benzaldehyde released 48 μ l. of oxygen; the Na_2CO_3 released 85 μ l. of oxygen; *i.e.*, the rate of oxygen liberation from benzaldehyde was 56 per cent of that liberated from Na_2CO_3 . The values for several experiments range from 45 to 60 per cent.

It has also been suggested that the rate of oxygen production from benzaldehyde should be at least as great as the rate of oxygen production from carbon dioxide. Obviously, however, this is not a necessary condition. The rate of oxygen production depends upon the rate at which the hydrogen produced from water can reach the reducible material. With an "unnatural" material such as benzaldehyde this rate may be any value whatsoever (conditioned by the dissociation constant of the benzaldehyde and the reducing enzyme and the diffusion to that enzyme) and need bear no relation to the rate of oxygen production from carbon dioxide. We wish to emphasize that the effects observed are not small nor is their rate extremely low.

SUMMARY

Using intact cells of *Chlorella pyrenoidosa* it is possible to obtain oxygen by the reduction of certain reducible materials other than carbon dioxide. Of these, benzaldehyde was studied in some detail. This reduction does not involve the production of carbon dioxide from the benzaldehyde. Stoichiometrical relationships as expressed by the following equation:



are somewhat difficult to obtain because the benzaldehyde can disappear from the reaction mixtures by dark reactions. The technique is now available which permits detailed studies of the oxygen-liberating mechanisms in photosynthesis.

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PHYSICAL PROPERTIES OF THE ALLANTOIC AND AMNIOTIC FLUIDS OF THE CHICK

III. SURFACE TENSION

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Up to the present time, little information has been available on the surface tension of the allantoic and amniotic fluids of the developing hen's egg. There seems little doubt but that water is the main constituent of both these fluids, and a study of their respective surface tensions throughout the incubation period of the egg should give some idea of the distribution, within these fluids, of the various dissolved substances. Because of volume considerations, the range studied was limited to that period of incubation between the 7th and 19th days.

The apparatus used was the familiar du Noüy tensiometer. Since the volumes of the liquids to be measured were often extremely small, a special platinum ring, which had a mean circumference of $1.48 (\pm 0.005)$ cm., was used instead of the standard 4 cm. size. Johlin (1928), in his criticism of the du Noüy method, points out that a considerable error is introduced when ring size is reduced. However, it was felt that this smaller ring would still give relative values which would be comparable, although slightly lower than those which would have been obtained had a standard ring been usable.

The eggs used all came from the same flock of Barred Plymouth Rock hens. They were incubated the desired time at 38.5°C . Samples of the two fluids were obtained in the manner previously described (Walker, 1943*a*). Immediately after removal from the egg, they were placed in small vials and stoppered. They were allowed to remain in these vials for 20 minutes before the measurements were begun. This afforded ample time for the liquids to cool down to the experimental temperature (25°C .). A measured portion of the sample was then transferred to the small watch crystal used in the tensiometer and left there 5 minutes before making the first determination. In these measurements, the procedure and precautions recommended by du Noüy (1926, 1929) were strictly followed. Three separate determinations were made on each sample tested and the average value taken as the relative surface tension. As du Noüy points out, this procedure is adequate when merely relative readings are desired. The individual values thus obtained are higher than when the "absolute technique" is employed, but they are quite comparable, one with another.

To calibrate the apparatus, the following substances were measured at the

experimental temperature: Distilled water, glycerine, 4M resorcinol, aniline, benzene, chloroform, and methyl alcohol. The tensiometer readings thus obtained were plotted against the surface tension of each liquid as given in the "International Critical Tables," and from the graph thus constructed, the surface tension value for any unknown liquid could be read off directly as soon

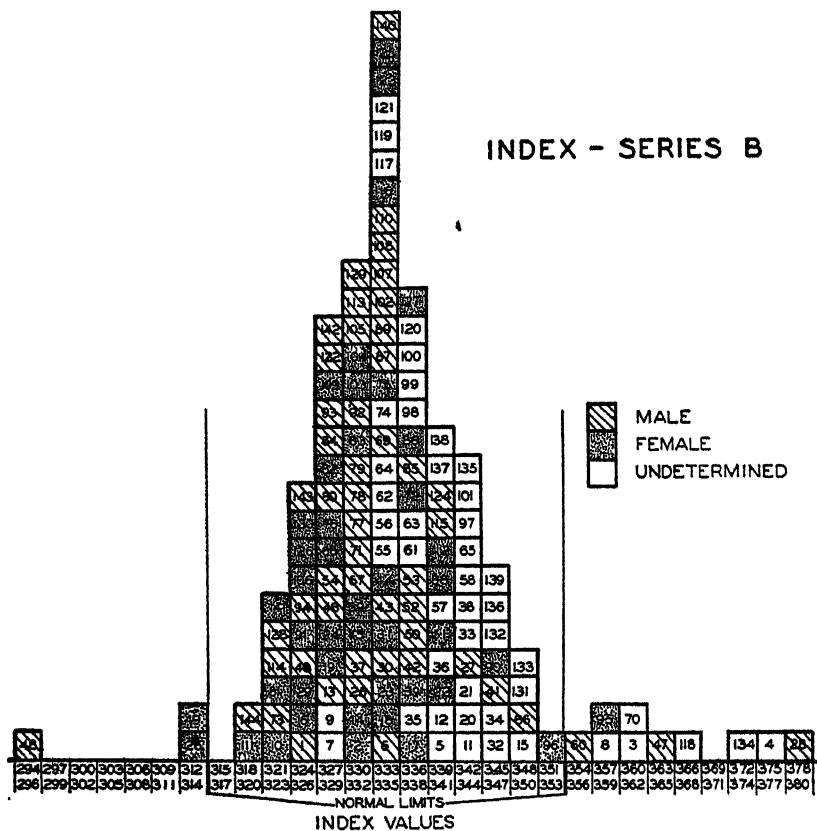


Fig. 1. Histogram resulting from application of index to experimental material.

as the average tensiometer reading had been determined. Determinations on distilled water and benzene (near the limits of the surface tension range studied) were repeated at frequent intervals throughout the course of the investigation. No significant changes were observed.

Since considerable variation was encountered in the weights of chicks of supposedly the same incubation age, incubation time was not used as a primary criterion of the degree of development, but the embryos were classified according to weight. In the accompanying data, an attempt has been made to clas-

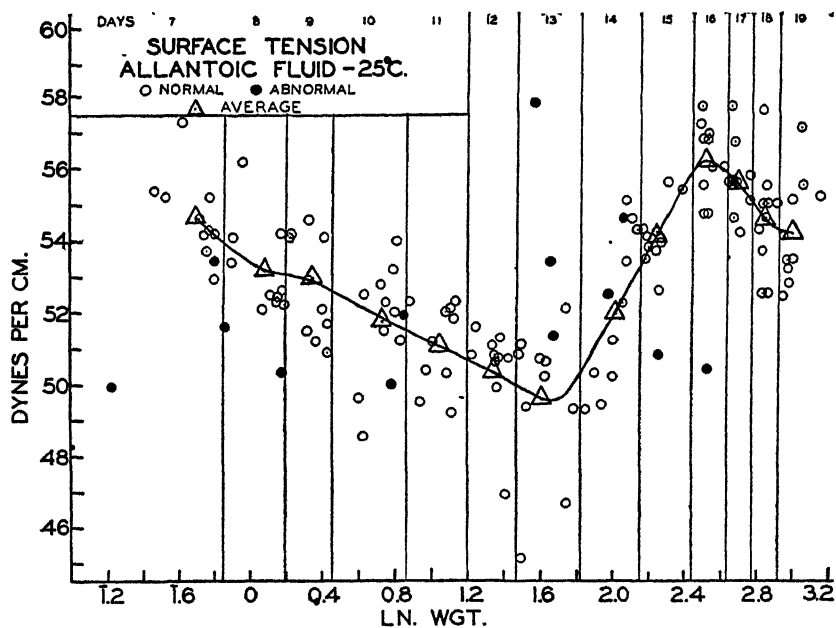


FIG. 2. Surface tension of the allantoic fluid (approximate incubation age indicated on top scale).

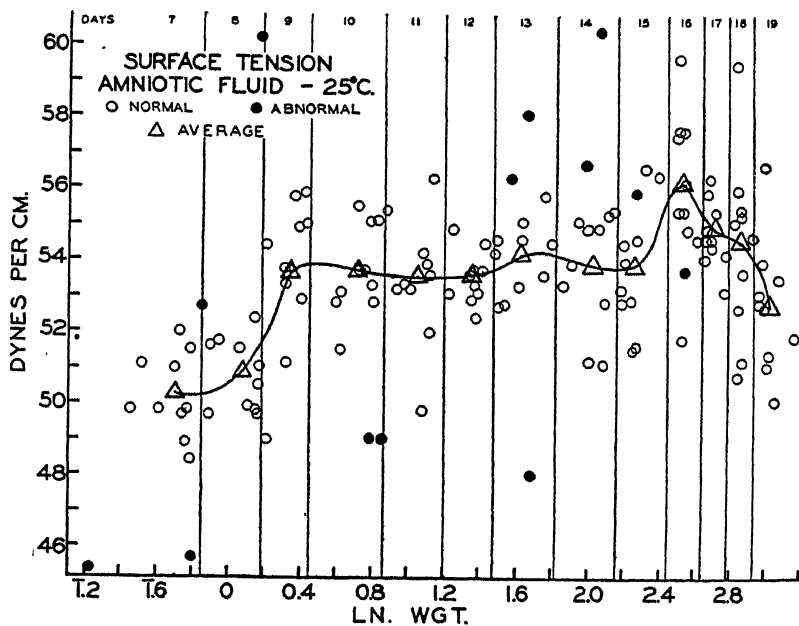


FIG. 3. Surface tension of the amniotic fluid (approximate incubation age indicated on top scale).

sify the animals roughly by days of incubation, but this is only an approximate, more or less arbitrary classification. The index of normal development previously described (Walker, 1938) was employed, and the histogram thus obtained (Fig. 1) is of the expected configuration, with the mean class represented by indices of 333 to 335. Embryos whose indices lay outside the range of normality (315 to 353) were discarded, although surface tension values for these individuals have been included in Figs. 2 and 3 for comparative purposes. In Table I is presented a brief summary of these experiments; the averages of each set of ten individual points (chicks of a certain weight group), are also plotted in Figs. 2 and 3.

TABLE I
Average Measurements of All Embryos Used

Age	Crown-rump length	Weight	ln. w.	Surface tension	
				Al. L.	Am. L.
<i>days</i>	<i>cm.</i>	<i>gm.</i>		<i>dynes per cm.</i>	<i>dynes per cm.</i>
7	2.6	0.74	1.70	54.7	50.3
8	2.9	1.08	0.08	53.2	50.9
9	3.3	1.41	0.34	53.0	53.7
10	3.8	2.08	0.73	51.8	53.8
11	4.2	2.85	1.05	51.1	53.6
12	4.7	3.89	1.35	50.4	53.6
13	5.2	5.06	1.62	49.6	54.2
14	5.8	7.52	2.02	52.0	53.9
15	6.4	9.46	2.25	54.1	53.9
16	7.0	12.42	2.52	56.2	56.2
17	7.4	14.67	2.69	55.6	54.9
18	7.8	17.14	2.84	54.6	54.6
19	8.3	20.17	3.00	54.2	52.8

DISCUSSION

The decrease in surface tension seen in the case of the allantoic fluid (Fig. 2) between the 7th and 13th days doubtless results in part from the general increase in nitrogenous compounds, such as proteins and uric acid, found during this period of Fiske and Boyden (1926). It may also indicate an increase in concentration of enzymes, for Berczeller (1913) has shown that the surface tension of various protein solutions decreases in the presence of ferments. The work of Przylecki and Rogalski (1927) has demonstrated the presence of uricase in the allantoic liquid of chick embryos from the 2nd to the 10th days of incubation, and it is quite possible that other enzymes may be present.

Following this period of decreasing surface tension values, there occurs a rise from definitely minimal values on the 13th day to as definite a maximum

on the 16th. This rise is quite puzzling in the light of present information on the composition of this fluid. Since the lowering of surface tension is a property of proteins in general, one might expect either that a decrease in the concentration of these substances is occurring here, or else that the relative proportions of water and inorganic materials increase. Although analyses of the chemical composition of this fluid are admittedly incomplete, several workers, among them Fiske and Boyden (1926), Targonski (1927), and Kamei (1928), have shown that there is a decided increase in the concentration of nitrogenous materials during this time. Also, since both Kamei (1928) and Yamada (1933 *a*) have found that the specific gravity of the allantoic liquid is increasing throughout this period, this observed rise in surface tension cannot be explained in terms of any increase in the proportion of water present. As for the inorganic constituents, conductivity measurements previously reported (Walker, 1943 *a*) indicate that the relative concentration of these substances is slightly on the decrease from the 13th to the 16th days of incubation.

The relation between the hydrogen ion concentration of a solution and its surface tension has received considerable attention in the past. Johlin (1930) has pointed out that the surface tension of gelatine solutions is decreased by the addition of small amounts of acid and increased in the presence of small amounts of base. Sugino (1929) working with solutions of amylamine, Yusawa (1935) studying the surface tension of human urine, and Gardner and Semb (1935) using various anesthetics have found a similar relationship to hold. However, Yamada (1933 *a*), using the hydrogen electrode, and Walker (1943 *b*), using the glass electrode technique, have both found diminishing pH values during this period of incubation, with the maximal decrease (Walker) occurring at precisely this period. This is exactly the opposite of what would be expected from the present results on the investigation of surface tension. The explanation of this pronounced rise in surface tension values must hence be sought elsewhere, possibly linked in some way to the fact that the change from mesonephric to metanephric excretion is occurring at about this stage of development.

The last few days of incubation studied reveal a decrease in surface tension values for the allantoic fluid which does agree with the investigations of hydrogen ion concentration referred to above. This might also be explained simply on the basis of the diminution in the water content of the allantois which is known to take place at this time. This would result in a marked increase in the relative amount of nitrogenous excretory residues. This is also borne out by the sharp decline in conductance values reported by Walker (1943 *a*).

With the exception, therefore, of this period between the 13th and 16th days of incubation, analysis of changes in the surface tension of the allantoic fluid yields results which are essentially in accord with what has previously been found from investigations of chemical and other physical properties. During this brief period, however, the surface tension of the allantoic fluid behaves in a

manner which is exactly the opposite of what one would expect and which still challenges explanation.

On the 7th day, in the case of the amniotic fluid (Fig. 3), the low surface tension values obtained contradict certain current theories as to the origin of the amniotic fluid; this has frequently been described as a sort of embryonic sweat, composed for the most part of water and inorganic substances. Such a mixture should show a much higher surface tension. The presence of substances which would materially lower surface tension is certainly not predictable from results of chemical analyses. Indeed, Fiske and Boyden (1926) reported only 2.9 mg. per cent total nitrogen on the 7th day, and Kamei (1928) but 11.9 mg. per cent on the 9th day. A suggestion as to the type of substance which might be responsible for these low initial values is perhaps afforded by the findings of Yamada (1933 *a*, 1933 *b*) that sugars are present in the amniotic fluid early in development; furthermore, Urbitch (1924), in demonstrating that the amniotic fluid can be recovered from the gut of the embryo as early in development as the 9th day, also suggests that nutritive substances (which would presumably tend to lower surface tension) may be present during these early stages as well as after the rupture of the sero-amniotic raphe. The marked increase in surface tension values between the 7th and 9th days is doubtless correlated with the increase in the volume of this fluid found by Yamada.

Between the 9th and about the 15th days, the relative constancy of the surface tension values for this fluid is interesting in view of the fact that the influx of protein material from the albumen sac begins on the 12th day (Hirota, 1894). That no marked changes in surface tension take place at this time must mean a rapid absorption of this albuminous material by the embryo. As a matter of fact, the change in surface tension, when it does occur (abruptly between the 15th and 16th days), is not of the nature of a decrease, as might be expected from the marked increase in protein material occurring at this time, but is, on the contrary, a rise to a maximal value. It will be remembered that a similar, but relatively greater, increase in surface tension takes place at this same stage in the case of the allantoic liquid. Inasmuch as no reliable chemical analyses of the amniotic fluid have been carried out during this period of development, it is impossible to say what rôle the inorganic constituents may be playing here. From the conductance values reported by Walker (1943 *a*), it is evident that the number of freely ionizing substances approaches a minimum at this time. Furthermore, since the total volume of the amniotic fluid is diminishing, no explanation may be made in terms of an increase in the relative amount of water present. The fact that the curves for both these extra-embryonic fluids rise to the same maximal value on the 16th day is interesting, but impossible of interpretation on the basis of existing information.

During the last few days of incubation studied, the surface tension of the amniotic fluid is decreasing, as would be expected in view of the large decrease

in volume occurring at this time. Another factor here may be the fact that excretory products may now be entering the cavity of the amnion by way of the cloacal opening. The presence of digestive enzymes during this period is quite possible, and these would also tend to reduce existing surface tension values.

The most striking general feature of the present results is that over the entire range of development studied, the values for the surface tension of these extra-embryonic fluids are considerably below 72 dynes per cm., the value for distilled water. Thus, any solutes would become concentrated in the peripheral portions of the liquid mass, or at the boundaries between these fluids and their respective enveloping membranes. In the case of the amniotic fluid, this means that dissolved substances will also become more concentrated at the surface of the developing embryo. Even inorganic salts will be found here, despite the fact that had they been present alone they would have behaved in just the opposite fashion. Their presence at the interphase boundaries together with the organic solutes (proteins, etc.) will further the precipitation and coagulation of these latter substances, a remark which readily admits of experimental verification, since at the end of incubation, the walls of both the allantois and amnion are lined with a copious cheesy precipitate. The close juxtaposition of these inorganic and organic solutes to the highly vascular allantoic membrane would unquestionably favor any processes of selective absorption which might be going on. In this connection, Needham (1931) has postulated a circulation of free basic radicals and ions in addition to the known circulation of water. In the amniotic fluid, this concentration of rich protein and inorganic material at the surface of the embryo may play a significant part in various morphogenetic and histogenetic processes.

The observed day-to-day variations in the surface tension may perhaps be interpreted as symptoms of variations in the rate at which such processes as selective absorption and deposition of protein precipitates are going on. According to this view, the increase in the surface tension of the allantoic liquid noted between the 13th and 16th days might indicate a temporary slowing down of these processes such as might well be occasioned by the transfer of excretory function from the mesonephros to the metanephros and attendant changes in the embryo's metabolism. Similarly, the rise in surface tension seen at approximately the same time in the case of the amniotic fluid might be explainable on the grounds of changed metabolic requirements. That these are not complete explanations in either instance must readily be admitted; they are put forward simply as statements provocative of further experimentation.

CONCLUSION

The study of the changes occurring during incubation in the surface tension of the two extra-embryonic fluids of the hen's egg has yielded results which in part harmonize with previous studies of the chemical and physical nature of

these liquids, and which in part are quite inexplicable in the light of these investigations. The necessity for a more thorough chemical analysis of these fluids is clearly indicated before any exact evaluation of their significance to the developing embryo can be made.

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POTENTIAL, IMPEDANCE, AND RECTIFICATION IN MEMBRANES

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INTRODUCTION

Since the early recognition of the value of electrical measurements on biological cells and tissues, the resting potentials of many cells have been determined and have been shown to vary with the degree of activity and with the composition of the external medium. When a steady current is passed, the potential takes on a new value. The ratio of the current to the change in potential, namely the conductance, has also been measured for many cells and has also been found to depend on physiological state and environment. Since ion motion is equivalent to an electric current, the conductance may be taken as an index of the "ion permeability."

The introduction, by Kohlrausch, of A.C. measurements in place of D.C. has allowed more reliable results to be obtained. Further, one is then able to determine not only the conductance, but the capacitance as well. The use of a range of frequencies of the measuring current (Höber, 1912; Gildemeister, 1919), has resulted in a picture of the cell membrane as electrically equivalent to a parallel resistance-capacitance combination, the capacitance having a constant phase angle less than 90° (Philippson, 1921; Cole, 1932).

If the magnitude and direction of the current is varied, it is found that the conductance may change. In particular, it may be large with the current in one direction and small with the current in the other and the system then shows electrical rectification. This phenomenon has been studied in *Valonia* (Blinks, 1930*a, c*), in the squid giant axon (Cole and Baker, 1941; Cole and Curtis, 1941), and in frog muscle (Katz, 1942).

According to the membrane hypothesis of Bernstein, the permeability of the cell membrane increases during activity. Corresponding changes in conductance have been found in many systems and it has recently become possible to study them quantitatively in *Nitella* and in the squid giant axon (Cole and Curtis, 1938, 1939). By contrast, the capacitances appear to be remarkably constant and independent of physiological state and environment. They have thus been interpreted as representing the dielectric properties of fixed elements of the membranes to which they are referred while the conductances and potentials depend primarily on ion mobilities and concentrations and their distributions.

In so far as any of these properties may involve physical rather than metabolic

factors, attempts have been made to duplicate them in artificial systems. Of these, collodion membranes have received the lion's share of attention. In addition to diffusion properties, potentials and conductances have been studied chiefly by Michaelis under a number of conditions (Michaelis, 1929). Blinks (1930 *b*) observed rectification in very thin membranes in certain combinations of environmental solutions, and Sollner and his coworkers (1940, 1941, 1943) have studied some relationships between potentials and structural characteristics. Teorell (1935) and Meyer and Sievers (1936) have pointed out the importance of fixed ions in the determination of potential properties. Structural investigations on biological membranes have proved difficult. However, it seems likely that they consist largely of protein and phospholipid and that there may be an organized arrangement of these elements.

In spite of the amount and variety of information at hand, there is, so far, only one system, the squid axon, on which potentials, conductances, and rectification properties have all been studied. Correspondingly, the analytical methods customarily adopted in interpreting the results are limited in their scope. Thermodynamics, for example, is quite inadequate for handling conductance and rectification phenomena. The kinetic method outlined by Planck (1890) appears capable of fulfilling the desired function. As ordinarily applied, however, it has proved only moderately successful in explaining the relations found in biological systems between the membrane potential and the composition of the medium. Several examples are cited by Steinbach (1940) of cells for which the logarithmic relation between potential and concentration does not hold.

The work to be reported here represents an attempt to obtain simultaneous measurements, in some simple artificial systems, of the electrical properties mentioned and to modify and extend the kinetic theory of ion motion to cover the observed phenomena to the extent that they may be physical rather than metabolic. This will involve a consideration of the fixed structural characteristics of the membrane and of the ionic configuration of the medium. Obviously radical simplifications will have to be adopted thereby vitiating the results somewhat. Nevertheless rough agreement may be hoped for in some cases and such an analysis may also help in clarifying the influence of some of the controlling factors.

Preliminary experiments were made with protein membranes prepared by the method of Dean (Dean, Curtis, and Cole, 1940) measuring the current and potential directly.

An I and J tube, each containing dilute KCl with a trace of tannic acid, were suspended from rack and pinion mounts in a beaker of butyl acetate so as to produce a pair of oil-water interfaces one over the other. Flecks of solid protein were placed on the interfaces and the system was allowed to stand for about a half hour for spreading and stabilization to occur. The J tube was then raised so as to press the interfaces

gently together and the system allowed to stand, this time for several hours. Thereafter a slow change in properties continued but the arrangement could be kept as long as 3 days provided that precautions were taken to avoid mechanical shocks and evaporation of liquid. Scrupulous care was required in handling glassware and solutions.

The best results were obtained with dried, powdered blood serum or egg albumin. It was not found possible to make stable membranes from any of several carefully purified proteins. The membranes could be made in various thicknesses depending on the amount of protein placed on the interfaces. Whether the butyl acetate played an important part in determining the properties of these membranes could not be ascertained.

The rectification observed in these membranes under moderate concentration gradients of salt was rather small indicating that a method of greater sensitivity was needed. The A.C. bridge described below was then introduced and was found quite satisfactory. However, the instabilities were thus magnified considerably. In addition only one pair of solutions could be used with any membrane so that no intercomparison of results was possible. Recourse was then had to thin, dried membranes of collodion to which cephalin or lecithin had been added, the phospholipid introducing a large number of fixed dipolar ions into the membrane. A few measurements were also made on membranes of collodion and of polystyrene, and on onion and tomato cuticles.

Impedances were then determined over a wide frequency range as a function of membrane current in various arrangements of environmental solutions (NaCl, KCl, HCl). Membrane potentials were also measured.

Material and Methods

The plastic membranes were prepared by the method used by Blinks (1930b). The materials were Mallinckrodt Parlodion, a commercial sample of polystyrene, which may have contained a plasticizer, Merck egg lecithin, and cephalin prepared from calf brain. The collodion was dissolved in alcohol-ether. The phospholipid desired was added to the solution. Most of the membranes studied were made from a solution containing 5 per cent collodion and $\frac{1}{2}$ per cent cephalin. Butyl acetate was the solvent for the polystyrene. A small amount of a solution of the material was pressed between two clean glass coverslips and allowed to dry thoroughly. On placing the coverslips in water, the membranes came loose and were then dried and stored between sheets of filter paper. The thicknesses as measured with a micrometer were from 2 to 8 micra.

The measuring cell is shown in Fig. 1. The impedance electrodes were platinized platinum bands 8 mm. wide by 10 mm. in diameter and sealed to the glass 2 mm. from the membrane. Constant current was supplied through a pair of chlorided silver wire coils placed 4 or 5 cm. from the membrane and supported by the stoppers which also held inlet and outlet tubes for running solution through the cell. The membranes were mounted directly between the ground glass lips of the tubes or first placed between a pair of celluloid washers of a convenient size. Of the perfusion tubes, one pair con-

nected each to a reservoir and the other pair to waste beakers. It was thus possible to control independently the solutions bathing the two sides of the membrane. Much of the data was, however, obtained with the electrode tubes used for the protein membranes which had a similar arrangement of electrodes but no facilities for perfusion.

The plant cuticles were stripped, boiled to destroy living tissue, and gently scraped to remove debris.

When impedance measurements without current flow were desired, another cell could be used which had large platinum disc electrodes. It is described in detail by Cole and Guttman (1942).

Measurements were made at room temperatures (20–25°C). The variation in any experiment was less than 1°C.

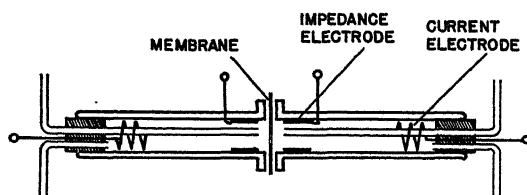


FIG. 1. Measuring cell for solid membranes. The small tubes are for perfusion, the upper pair connecting with reservoirs and the lower pair acting as waste outlets.

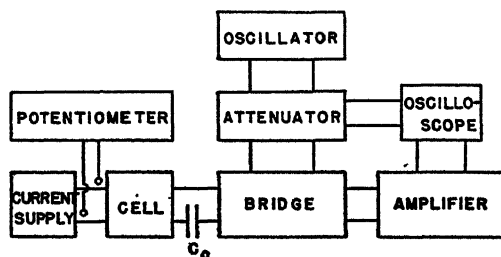


FIG. 2. Block diagram of measuring equipment.

Apparatus

The measuring equipment (Fig. 2) consisted of:

1. A grounded point, a. c. Wheatstone bridge including a Leeds and Northrup "Campbell-Shackleton" ratio arm assembly.
2. A Western Electric telephone type oscillator adjusted for a frequency range of 35 cycles to 100 kc. and a harmonic content of 1 per cent.
3. An attenuator for controlling the input to the bridge.
4. A 4-stage output amplifier, resistance-capacity coupled except for the second stage which could be tuned to the measuring frequency. This was necessary in order to maintain adequate sensitivity by cutting down noise and harmonics.
5. A 3 inch oscilloscope as an indicator. The amplified bridge output was applied to the vertical plates and a portion of the oscillator output applied directly to the

horizontal plates thus providing a 1-1 Lissajous figure which at bridge balance 'was a horizontal line.

The "unknown" arm of the bridge contained, in addition to the membrane cell, a pentode circuit for supplying constant current independent of membrane and electrode impedances and through a very high internal impedance (10 megohms in parallel with $35\ \mu\text{mf}$). The blocking condenser, C_0 , served to keep the membrane system open-circuited and to prevent D. C. from entering the bridge circuit. It was large enough ($21.9\ \mu\text{f}$) to have a very small impedance except at the lowest frequencies.

A Leeds and Northrup K-2 potentiometer could be switched in to determine the potential difference between the silver chloride electrodes to within 0.2 mv.

The sensitivity of the bridge was about 0.05 per cent over most of the useful range with a falling off at the extremes of frequency and impedance. Since the "unknown" arm of the bridge may be represented as in Fig. 3, it will be seen that to obtain the equivalent impedance of the membrane a rather complicated set of calculations is, in general, necessary. However, the high value of the current supply circuit and the

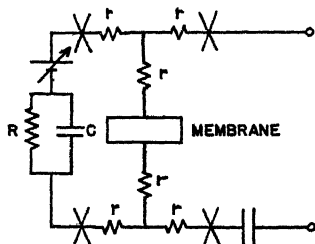


FIG. 3. Equivalent circuit of membrane, cell, and accessories. R and C represent the current supply circuit including stray capacities. Resistances r are for the solution. The X 's are electrode impedances.

low values of the electrodes and blocking condenser allowed, for most of the membranes, the neglecting of these corrections and impedance values were then readily obtained which were accurate to about 1 per cent.

EXPERIMENTAL

For purposes of analysis, a large number of measurements were required on a single membrane. It is therefore pertinent to discuss the experimental procedure in some detail. The concentrations of the environmental solutions were chosen largely with an eye to bringing the resistances within the optimum range of the bridge (10^3 to 10^5 ohms). This usually resulted in the use of 0.01 to one molar solutions approaching the condition found in biological systems rather than the high dilution which has been found most useful in electrochemical studies. Also, the areas and thicknesses represented a compromise between the factors of uniformity, mechanical strength, and impedance values.

When the membranes were clamped in position and the solutions added to the cell, some time was required for the impedance to reach a steady value.

With fresh, dry membranes this was 2 or 3 hours, varying with the type and thickness of the membrane used. Replacement by another solution was followed by a more rapid attainment of a steady value. If the same solution bathed both sides it was occasionally replaced before beginning the measurements. If different solutions were used, a continuous flow of solution was kept up ($\frac{1}{2}$ to 1 cc./minute) to maintain as nearly as possible constant boundary values of the concentrations. However, the effectiveness of such low rates of flow is questionable and it seems worth while to attempt an estimate of the diffusion rate of salt when there is no perfusion.

The diffusion rate, S , in mols/cm.²-second is related to the concentration difference across the membrane by the equation

$$S = \alpha \frac{n' - n^0}{a}$$

where α is the diffusion constant, n' and n^0 are the membrane concentrations at the two sides, and a is the thickness. Also the mobility $u = \frac{F}{RT} \alpha$ where F , R , and T have their usual significance of Faraday, gas constant, and absolute temperature. Then the relation may be written.

$$S = \frac{RT}{F^2} \left(\frac{uFn'}{a} - \frac{uFn^0}{a} \right)$$

But $\frac{unF}{a}$ is the conductance, Λ , of the membrane (per cm.²) at a salt concentration n . Hence

$$S = 2.5 \times 10^{-7} (\Lambda' - \Lambda^0)$$

Λ , however, is known. At a concentration of 1 molar in the adjacent solution, it is usually less than 10^{-3} mhos/cm.² and decreases with dilution. Thus $S < 10^{-10}$. If the solution on the other side is at 0.1 molar, this means an increase of 0.1 per cent in its concentration during the 1st second. Reference to Fig. 4 shows that the estimate is roughly consistent with the observed changes. It will also be seen that changes in conductance with current are much less affected. Hence, except with membranes of high conductance under a large concentration gradient, perfusion does not appear to be of fundamental importance within the limits of reproducibility attainable.

The most important limitation encountered was that due to prolonged soaking of the membranes most of which appeared to undergo a slow, steady dissolution resulting, perhaps, from swelling or from loss of phospholipid or other material. There was a gradual increase in conductance which could amount to as much as 20 per cent in 24 hours. While this was relatively unimportant for a single set of impedance-frequency or impedance-current data, it destroyed

any hope of obtaining more than an approximate intercomparison of results such as is required for any extended analysis of the membrane characteristics.

The impedance measurements were made with a very small bridge input corresponding to 1 or 2 microamperes through the membrane. Compared with the polarizing currents of up to 30 microamperes and in view of the magnitudes of the conductance changes, this is small enough for the impedances so determined to represent quite well the slope of the current-voltage curve of the membrane at the "operating point" specified by the steady current. It is also small enough to avoid any interference of the non-linearities with the bridge balance. A set of data could usually be obtained in about 15 minutes, the 1 kc. values being checked after the run to make sure that no large drifts had occurred.

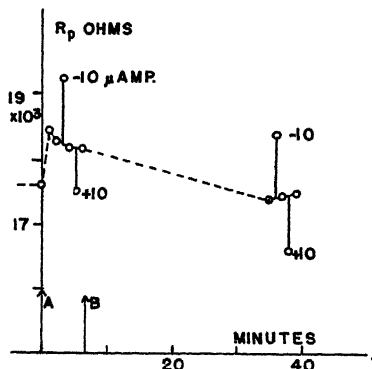


FIG. 4. Changes of membrane resistance with flow of solution. Collodion-cephalin membrane. Left hand side of membrane in 0.1 N KCl, right side in 1 N KCl. At A the flow was started, at B it was cut off.

The impedance-current runs were taken with the D.C. in alternate directions and flowing only long enough to allow rebalancing the bridge. This served both to minimize residual accumulation effects and to maintain the current supply electrodes in a symmetrical condition.

While the current supply circuit had a capacity of more than 1 milliampere, it was found that if the applied field in the membrane exceeded a few thousand volts per centimeter there was a tendency for the membrane conductances to undergo irreversible changes. Accordingly the results obtained here correspond to a small part only of the entire current-voltage curves of the membranes and preclude the attainment of any limiting conductance values. However, the intrusion of possible Wien effect was thereby ruled out as were also any effects due to heating.

The blocking condenser, C_0 , provided a convenient alternative to a counter E.M.F. in series with the membrane. It also acted as a delay device to the

passage of D.C. through the membrane. When the membrane resistance changed with current flow, the rise in current through it on switching in the supply circuit was distorted from the simple, exponentially delayed form although the time to reach a practical steady state was not usually altered very much. This effect of C_0 could be checked by changing its value or by shorting it out altogether. In the latter case it was necessary to correct for the current lost into the bridge network. It was then found that except for membranes of the very highest resistance the steady state was reached too rapidly to be seen on the oscilloscope face. This indicated that the "time constant" was less than 0.1 second. The procedure was also useful in showing the absence of slow accumulation effects of current flow as it served to spread out on a linear time scale the impedance-current curve on the oscilloscope.

In general the non-linearities of the membranes studied were not large and it was necessary to make sure that the apparatus itself did not contribute to any of the effects seen. When the membrane cell was replaced by a conventional R-C network, and the behavior of the electrodes and solutions observed without the membrane, it was found that within the range of measurement used such effects were negligible. Two further points should be made. First, with the A.C. equipment used, steady potentials have no effect on the bridge balance. Second, the solution resistances are so much smaller than the membrane resistance that changes in the former due to current flow are very small. It follows that any impedance variations observed must be due to changes within the membrane itself.

Membrane potentials were determined from time to time. As the membranes were allowed to stand in solution, the potentials underwent slow, irregular changes of a millivolt or two so that the same limitation applies here as to the impedance measurements. At the end of each experiment control runs were made.

The impedances were recorded as parallel resistance, R_p , and capacitance, C_p . For ease in handling corrections and for convenience of plotting, the data were frequently converted to the equivalent series resistance and reactance using the formulae

$$r_s = \frac{R_p}{1 + (R_p C_p \omega)^2}$$

$$x_s = - \frac{R_p^2 C_p \omega}{1 + (R_p C_p \omega)^2}$$

Elimination of ω between these two equations leads to the impedance locus while elimination of R_p leads to a locus showing the path followed by the impedance when the resistance varies. Since C_p and ω occur together, the impedance locus is also the locus for varying capacitance (Cole, 1932; Cole and Curtis, 1938).

RESULTS

When the same solution bathed both sides of the membrane and in the absence of current flow, the observed impedance properties were entirely consistent with the results already obtained by other investigators.

1. The membranes behave like parallel resistance-capacity combinations.
2. The capacitances have a phase angle less than 90° and independent of frequency. For the protein membranes the values found were $65-75^\circ$, for collodion-lecithin $79-82^\circ$, for collodion-cephalin $84-86^\circ$, for collodion alone $88-89^\circ$, and for onion cuticle $83-85^\circ$.
3. The conductances are roughly proportional to those of the environmental solutions (*cf.* Green, Weech, and Michaelis, 1929) but are much smaller, although somewhat larger than the estimated values for the membrane material in bulk. For example, conductances in mhos/cm.² in 0.01 normal KCl were: for protein membranes 10^{-2} – 10^{-4} , for the plastics 10^{-6} – 10^{-8} , depending on the thickness and for onion skin about 5×10^{-5} . Bulk nitrocellulose has a specific conductance* of 10^{-10} – 10^{-11} mhos/cm.
4. The capacitances vary slightly from one solution to another. The dielectric constants are somewhat larger than those of the bulk membrane material. As to values in $\mu\text{f}/\text{cm}^2$ at 1 kc. we find for the protein membranes 0.005–0.5, for the plastics 5×10^{-4} – 8×10^{-3} , for onion cuticle 0.1–0.2. Bulk nitrocellulose has a dielectric constant* of about 8.
5. The phase angles show little if any variation with kind or concentration of salt.

The above statements should be qualified somewhat. The membranes containing phospholipid and those made of dried blood serum showed a slight but definite tendency to depart at the lowest frequencies from the simple impedance loci (circular arc) in such a way as to indicate the presence of a low frequency component beyond the range of the bridge (*cf.* Fig. 5). Since phospholipids and proteins are known to have large electric moments (Hausser, 1935; Oncley, 1940) and since these materials are rather heterogeneous, this is not surprising. However, it emphasizes the danger of interpreting extrapolated zero frequency values obtained from loci as the actual D.C. resistances of the membranes. In any case the extrapolated value may be regarded as representing a resistance component whose variations are to be considered.

Nothing is known of the values or variations of partition coefficients and, in view of the deterioration of the membranes, the relation between membrane and solution resistances cannot be further analyzed at present.

When the two sides of the membrane were in solutions of different salts or different concentrations of the same salt, the observed impedances were in

* Handbook of chemistry and physics, Cleveland, Chemical Rubber Publishing Company, 24th edition, 1940.

between the values seen in either solution alone. The mode of variation was not a direct function of concentration or conductance and will be considered later.

The membrane impedance varies with the current; and the variation is such that it can be interpreted as a change in conductance only. The parallel capacitances as read on the bridge showed very little change and, since the corrections are small, these values are close to the actual equivalent capacitances. The loci show that the impedance changes with current flow follow closely the theoretical arc for a resistance change only (Fig. 5). Furthermore since the equivalent circuit can be represented as a membrane conductance in parallel

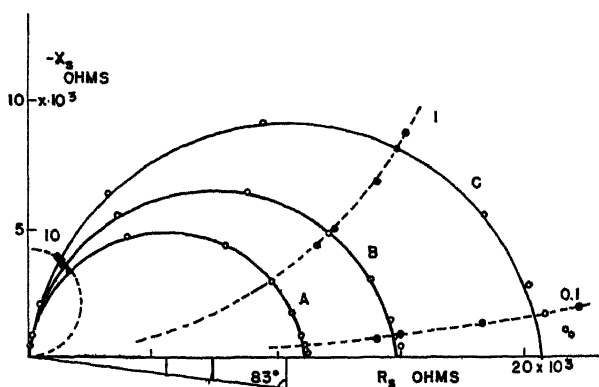


FIG. 5. Impedance loci for a collodion-cephalin membrane in three arrangements of KCl. Curve A, 1 N on both sides; B, 0.3 N on left, 1 N on right; C, 0.1 N on left, 1 N on right. Open circles are locus points with no current flow; solid circles, ± 15 microamperes. Numbers outside large arc refer to measuring frequency in kilocycles. The dashed arcs are theoretical loci for changes in resistance only. All data taken within 48 hours.

with a membrane capacitance having a constant phase angle, and since the series conductance of the adjacent solution is very much larger than the membrane conductance, it can be shown that, except at very high frequencies, the conductance changes with current are independent of frequency. Within the limits of reproducibility of the data, this was found to be true. One is thus allowed the use of a single frequency in making impedance-current measurements. Most of the data were obtained at 1 kc.

A set of conductance-current curves is given in Fig. 6 for a collodion-cephalin membrane in several arrangements of KCl and more complete data on this membrane are given in Table I. This behavior is typical of these membranes. At higher currents, however, there was frequently a tendency for the high conductance side of the curves to show a reversal. Unfortunately the onset of irreversible changes makes these measurements unreliable.

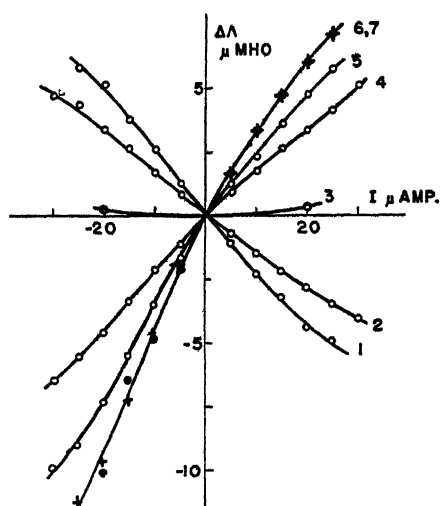


FIG. 6. Conductance as a function of current in a collodion-cephalin membrane for different concentration ratios of KCl. The concentration on the right hand side of the membrane was kept fixed at 0.2 N and that on the left side was varied from curve to curve. Ratio r is concentration on right hand side of membrane to that on left hand side. For curve 1, $r = 0.2$; curve 2, 0.4; curve 3, 1; curve 4, 2; curve 5, 3; curve 6, 5; curve 7, 7. No perfusion. All data taken within 30 hours. Conductance values for no current and no concentration gradient; 82.5 micromhos initially, 98 micromhos at end of experiment. The positive direction of the current is from left to right through the membrane. For further data on this membrane see Table I.

TABLE I

Collodion membrane containing 10 per cent cephalin. KCl solutions. Right hand side 0.2 N, left side variable. Area 0.8 cm.² Thickness 3×10^{-4} cm. Sign of potential is that of left side with respect to right side as measured in external circuit.

$$V_0 = V - 58 \log r$$

Conc. ratio right/left	Specific conductance	ϵ at 1 kc.	$\frac{d\Delta}{dI}_0$ per volt	V total	V_0 membrane
	mhos/cm.			mv.	mv.
0.2	8.4×10^{-8}	21	-0.24	-37.5	3.1
0.4	5.4	20	-0.17	-19.8	3.3
1.0	3.3	18	0.0	0.0	0.0
2	2.6	18	0.20	15.5	-1.9
3	2.2	18	0.29	25.5	-2.2
5	1.9	18	0.38	37.5	-3.1
7	1.8	19	0.37	44.5	-4.6

In some systems the reversal occurred at smaller current values. An onion cuticle in HCl gave the curve shown in Fig. 7. This also happened with some of the collodion membranes especially after long soaking. The data on the latter membranes and on those from the polystyrene sample are incomplete, but suggest that a somewhat more complicated situation exists, especially with respect to the variation of rectification properties with concentration gradient. Even greater complexities were sometimes seen in the plant cuticles.

When different chlorides bathed the two sides of the membranes similar variations were found. The non-linearities were, however, usually greater even though the concentrations or conductances of the two solutions might be the same. In general, the amount of rectification appeared to vary with the membrane potential rather than with concentration or conductance gradient.

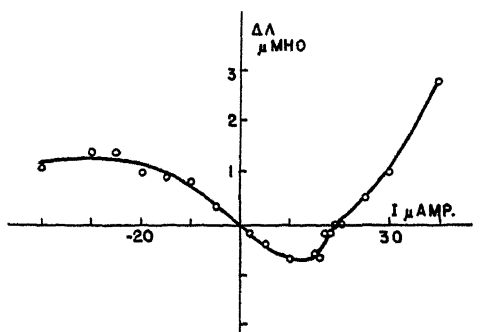


FIG. 7. Change in conductance with current in an onion cuticle in HCl, 0.01 N on left side of membrane, 0.1 N on right side. Conductance at no current, 132 micromhos. Positive current flows from left to right through membrane.

The potentials as measured were the sums of the membrane and electrode potentials. In most cases these total potentials were not far from the calculated concentration potentials of the electrodes. This, in addition to the drifts, made the membrane potential values quite unreliable. Nevertheless, there appeared to be a definite departure, especially in the membranes containing phospholipid, from the proportionality seen in solution between potential and the logarithm of the concentration ratio. It is, of course, impossible at present to make activity coefficient corrections for the membranes. Data for three membranes are given in Fig. 8.

It is evident from the foregoing that the results are not yet sufficiently complete or reproducible to justify more than a first approximation in an analytical treatment of the problem of the current flow in such systems. The experimental results may be summarized as follows. The smallness of the membrane potentials does not allow a definite statement as to whether or not the logarithmic relation between potential and concentration gradient is followed.

These membranes are thus quite non-selective in their action contrasting with many of those studied by Michaelis. The difference may be due either to the presence of the phospholipid plasticizers or to the thinness of the membranes. Sollner and Carr (1943) have shown that concentration potentials in collodion membranes decrease as the membrane is made thinner.

The conductances are proportional to those of the solutions and are small enough to suggest that the membranes have a relatively tight packing of structural elements. The dielectric constants then suggest a definite effect of the phospholipid. As to the rectification, the conductances appear to vary di-

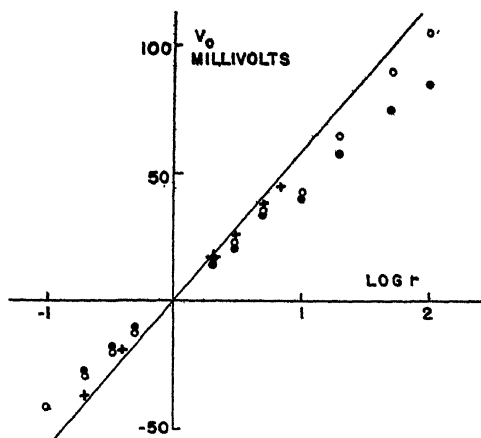


FIG. 8. Potential of membrane plus electrodes *vs.* log of concentration ratio r . Crosses, collodion-cephalin in KCl; open circles, collodion-cephalin in NaCl; solid circles, collodion in NaCl. Sign of potential is that measured in external circuit. The actual membrane potential is the difference between the total potential and the electrode potential calculated from the simple Nernst formula as given by straight line. The slope of the line is 58 mv. per 10:1 ratio but would be somewhat less if activity coefficients in solution were included.

rectly with the current within the range studied and for the collodion-cephalin membranes at least. Taking the initial slope of the conductance-current curves as an index of the amount of rectification, this is found to increase with the membrane potential. For larger currents, other factors may enter and in some types of membrane may obscure the simpler behavior by appearing at small current values.

Theory

Consider a solid membrane, of thickness a , immersed in solution and containing ions moving under the combined influence of diffusion and electrical forces. The membrane is assumed to be a uniform system having a dielectric

constant ϵ , and in which the ion mobilities and activity coefficients are constant. Let V be the potential at a point x ; n_i , the concentration of the i th ion; z_i , its algebraic valence; and $z_i u_i$, its mobility. Then the contribution, j_i , to the total current density, J , is given by the relation

$$j_i = -z_i u_i RT \left(\frac{dn_i}{dx} + z_i n_i \beta \frac{dV}{dx} \right) \quad (1)$$

where

$$\beta = \frac{F}{RT}$$

The potential distribution is related to the net charge density ρ through Poisson's equation

$$\frac{d^2 V}{dx^2} = -\frac{4\pi}{\epsilon} \rho \quad (2)$$

ρ being expressed in terms of the ion concentrations, fixed as well as mobile.

$$\rho = F \left(\sum z_i n_i + \bar{N} \right) \quad (3)$$

where the summation extends over the mobile ions and \bar{N} is the contribution of the fixed ions.

The assumptions call for some comment. The membranes dealt with consist of large molecular elements some of which may be ions. There appears to be a rather tight packing. If, then, the membranes are to be regarded as having a sieve-like structure, the "holes" must be almost of molecular dimensions (*cf.* Michaelis, 1929). It therefore seems reasonable to suppose that the current carriers pass through more or less randomly distributed interstices in the structure which is thus assumed uniform normal to the direction of flow. For many biological membranes this assumption is made in the absence of evidence to the contrary. There is less ground for postulating uniformity in the direction of flow. The method of preparation of the artificial membranes suggests that there may be oriented layers at the surfaces. Moreover, biological membranes may be highly organized or have a periodic structure.

The influence of the dielectric constant may be great. If ϵ is small, ion association is promoted (Bjerrum, 1926) and the Wien effect augmented (Onsager, 1934) although the presence of a water shell around the ions would tend to counteract this. However, for the present purpose, we have only to assume ϵ constant. Mobilities and activity coefficients actually depend on concentration. For ranges which are not too large, their constancy may reasonably be postulated.

We must also adopt simple boundary conditions. Since the system as a whole is electrically neutral,

$$\int \rho \, dx = 0$$

and if one assumes that the influence of external surface charges is small, the limits of integration are the boundaries of the membrane. Further, there must be a point at which $\rho = 0$, such that the total charge on one side of it, equal and opposite to that on the other, represents the equivalent condenser charge on the membrane, whether it be confined to the surfaces or distributed throughout. Evidently boundary layers may play a considerable part especially in the determination of the resting potential of the system. We shall, however, neglect them entirely in order to keep the problem in relatively simple form. The values of the concentrations and potentials at the boundaries will be considered as fixed, and the surface charge as zero.

We now have a set of equations and conditions from which, ideally, the current-voltage relation of the membrane may be derived. This set is not integrable in closed form as it stands however, and we are forced to introduce still further simplification if usable results are to be reached. The nature of the equations does suggest that, in general, a linear relation will not occur and that rectification will be more evident the greater the asymmetry of mobilities and concentrations. These same factors also determine the membrane potential and so the finding that the initial slope of the conductance-current curve increases with the membrane potential may be expected in any case.

Since equations (1) are linear in n_i , an explicit expression for the field distribution will lead directly to an equation for J . Practically, this means that we must find a simple treatment for equation (2). This appears to be possible in two ways. First, if there are no fixed ions present and if we assume microscopic electroneutrality, *i.e.* $\rho = 0$ everywhere, we have the situation dealt with by Planck in studying liquid junction potentials. However, the equations may also be solved for a finite fixed ion concentration, \bar{N} , and although we shall not make use of the results in the general form, it is of some interest to see how the presence of the fixed charges affects the various distributions. We consider univalent ions only.

Write equations (1) for each ion, divide each through by the corresponding mobility, and add separately the positive and negative contributions. Then

$$-\sum + \frac{j_i}{u_i RT} = \frac{d}{dx} \sum + n_i + \beta \sum + n_i \frac{dV}{dx}$$

$$\sum - \frac{j_i}{u_i RT} = \frac{d}{dx} \sum - n_i - \beta \sum - n_i \frac{dV}{dx}$$

Add and subtract these, introduce equation (3) remembering that $\rho = 0$, and let

$$\sum +n_i + \sum -n_i = N$$

(the total concentration of mobile ions). Then

$$\frac{dN}{dx} - \beta \bar{N} \frac{dV}{dx} = B \quad (4)$$

$$\beta N \frac{dV}{dx} = -gB \quad (5)$$

where B and g are given from the previous equations but are to be determined from the boundary conditions.

(4) integrates at once to

$$N - \beta \bar{N} V = Bx + \text{constant} \quad (6)$$

We now introduce the limits

$$\text{Let } N = N^0 \text{ and } V = 0 \text{ at } x = 0.$$

$$N = N' \text{ and } V = -\Delta V \text{ at } x = a, \quad N' - N^0 = \Delta N$$

Then

$$B = \frac{\Delta N + \beta \bar{N} \Delta V}{a} \quad (7)$$

Eliminate $\frac{dV}{dx}$ between (4) and (5) and integrate.

$$\beta V = -g \ln \frac{N - g \bar{N}}{N^0 - g \bar{N}} \quad (8)$$

and

$$\beta \Delta V = g \ln \frac{N' - g \bar{N}}{N^0 - g \bar{N}} \quad (9)$$

which determines g .

We may now return to equations (1), substitute from (5) and (8) to eliminate x and N , integrate, and rearrange.

Then, finally,

$$j_i = \frac{u_i RT}{a} (\Delta N + \beta \bar{N} \Delta V) (g - z_i) \frac{n'_i e^{-z_i \beta \Delta V} - n_i^0}{N' e^{-z_i \beta \Delta V} - N^0 - z_i \bar{N} (e^{-z_i \beta \Delta V} - 1)} \quad (10)$$

where $z_i = \pm 1$ and, of course, $J = \sum j_i$. Certain features of this result are of interest. When $\bar{N} = 0$ (the Planck case), the potential distribution is

logarithmic and the total concentration is linear although the individual ion concentrations are not. If \bar{N} is not zero, the potential distribution is more complicated and the total concentration distribution is no longer linear. Evidently the current-voltage relation is, in general, non-linear as well. One might also be tempted to speculate on the biological implications of such a curve were it justified either by experimental work or by the nature of the analytical simplifications. We shall consider only the case where $\bar{N} = 0$, but before doing this we take up an entirely different method of handling the differential equations.

We assume that the membrane contains a large number of dipolar ions near the isoelectric point and that these can act to minimize distortion of the field especially at low currents. We then approach a situation in which the field is constant and are led to a solution analogous to that given by Mott (1939) for electronic conduction in the copper-copper oxide rectifier. If the field is constant, equations (1) may be integrated directly and the limits introduced. Then

$$j_i = \frac{u_i F}{a} \Delta V \frac{n'_i e^{-z_i \beta \Delta V} - n_i^0}{e^{-z_i \beta \Delta V} - 1} \quad (11)$$

We may now proceed to indicate some of the consequences of the two types of treatment as being very rough approximations but as being simple enough to yield readily workable relations with experiment.

Consider a single electrolyte with a concentration gradient across the membrane. Then equation (10) reduces to an expression of Ohm's law, and we obtain

$$J = \frac{F}{a} (n' - n^0) \left[(u_+ + u_-) \frac{\Delta V}{\ln r} - \frac{u_+ - u_-}{\beta} \right] \quad (12)$$

where

$$r = \frac{n'}{n^0}$$

Further, if $J = 0$, we get the usual Planck formula for the liquid junction potential

$$V_0 = \frac{1}{\beta} \frac{u_+ - u_-}{u_+ + u_-} \ln r \quad (13)$$

Correspondingly, we obtain from equation (11)

$$J = \frac{F}{a} \Delta V \frac{(u_+ n^0 + u_- n') - (u_+ n' + u_- n^0) e^{-\beta \Delta V}}{1 - e^{-\beta \Delta V}} \quad (14)$$

and

$$V_0 = \frac{1}{\beta} \ln \frac{u_+ n' + u_- n^0}{u_+ n^0 + u_- n'} \quad (15)$$

The Planck equation (12) then predicts no rectification at all and a linear relation between V_0 and $\ln r$ while the constant field assumption yields relations which are non-linear in both cases.

Another situation arises when the membrane separates two different electrolytes having a common ion. Then both hypotheses result in non-linear current-voltage curves. If the total concentration is the same on both sides, the two are identical. This is to be expected since it is then possible for ions of one sign to replace each other freely without distorting the field.

The constant field case has the advantage that a general expression may be given, for any salt combination, by writing

$$\Lambda_+ = \frac{F}{a} [\sum_+ u_i n_i^0 + \sum_- u_i n_i']$$

and

$$\Lambda_- = \frac{F}{a} [\sum_+ u_i n_i' + \sum_- u_i n_i^0] \quad (16)$$

Then

$$J = \Delta V \frac{\Lambda_+ - \Lambda_- e^{-\beta \Delta V}}{1 - e^{-\beta \Delta V}} \quad (17)$$

Evidently, Λ_+ and Λ_- represent limiting conductance values for large potentials in one direction or the other. Also,

$$V_0 = \frac{1}{\beta} \ln \frac{\Lambda_-}{\Lambda_+} \quad (18)$$

For comparison with experimental data, we want Λ and $\frac{d\Lambda}{dJ}$ at $J = 0$. By differentiation,

$$\Lambda_0 = \beta V_0 \frac{\Lambda_- \Lambda_+}{\Lambda_- - \Lambda_+} \quad (19)$$

$$\left. \frac{d\Lambda}{dJ} \right|_0 = -\beta L \left(\frac{\beta V_0}{2} \right);$$

$$L(x) = \operatorname{ctnh} x - \frac{1}{x}$$

or, for V not too large,

$$\left. \frac{d\Lambda}{dJ} \right|_0 = -\frac{\beta^2 V_0}{6} \quad (20)$$

The corresponding Planck expressions for the single electrolyte are

$$\begin{aligned}\Delta_0 &= \frac{F}{a} (u_+ + u_-) \frac{n' - n^0}{\ln r} \\ \frac{d\Delta}{dJ} &= 0\end{aligned}\tag{21}$$

DISCUSSION

Having obtained explicit expressions for the membrane potential and for the conductance and its variation with current, we are in a position to see how far the data justify the above simplifications. First as to the membrane potentials. Equation (15) predicts a limiting value of the potential when the concentration ratio becomes very large or very small. It can be seen from Fig. 8 that the membrane potentials of the artificial systems are quite small. Then the ion mobilities are nearly equal and there is little difference between the two theoretical treatments. The results are not accurate enough to allow conclusions to be drawn.

On the other hand, the giant axon of the squid, *Loligo pealii*, in common with many biological membranes, appears to allow K^+ to pass much more easily than other ions. On the basis of our previous assumptions, we use data given by Curtis and Cole (1942 and unpublished) where the membrane potential was measured directly in solutions equivalent to sea water in which the sodium was replaced by varying amounts of potassium. The 10 mv. liquid junction correction was made as given in the paper cited and a small correction for a sea water-experimental solution junction was ignored. We may then calculate a mobility ratio $m = u_-/u_+$ on either theoretical picture, since both yield

$$\frac{dV_0}{d \ln r} = \frac{1 - m}{1 + m}$$

near $V_0 = 0$, and so obtain a complete curve. Fig. 9 shows such a curve together with the experimental data. Further, the value of r then found at the resting potential is 34. From chemical analysis, Bear and Schmitt (1939) give $r = 26$, and Webb and Young (1940) give $r = 29$ for the closely related species, *Loligo forbesi*. The data cited by Steinbach (1940) on potential variations with environmental K^+ concentration, appear to be of the same type.

The low value of 0.06 for the mobility ratio is consistent with the assumptions. Osterhout (1930) has found an even lower value (0.012) in *Nitella*. For such a ratio, enormous values of r would be required to produce any great deviation of the theoretical curve (15) from the linear relation.

The variation of conductance, at no current, with concentration gradient

might also be used for comparison. If, in equations (12) or (14), we take out a factor

$$A^* = \frac{F}{a} n^0 (u_+ + u_-)$$

this should be independent of r . However, this procedure is subject to serious interference from drift effects both for the artificial membranes and for the squid axon.

When we turn to the interpretation of the rectification properties, we find the Planck treatment useless since for the single salt it predicts no rectification

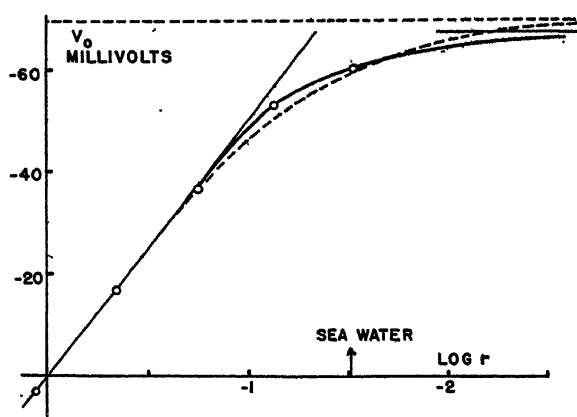


FIG. 9. Membrane potential of squid axon as a function of the ratio r of external to internal K^+ concentration. Circles and solid lines are experimental data. Dashed line is theory for $u_-/u_+ = 0.064$. Straight line is Planck equation.

at all and for the equal concentration case of two different salts it reduces to the same form as the constant field equation.

Equation (20) is a linear relation between the membrane potential and the initial slope of the conductance-current curves. Fig. 10 shows that for the artificial membranes this relation is followed only to an order of magnitude.

Blinks' (1930 *b*) results on collodion membranes for two different salts seem to fit the general picture rather well although his data are given in terms of D.C. measurements.

The only biological system on which adequate measurements have been made is, again, the squid axon (Cole and Curtis, 1941, and unpublished) and here also there is the difficulty of obtaining enough simultaneous data. There are several ways in which a comparison may be made. The ratio of limiting conductances may be put into equation (18) to get V_0 . This yields values of 70 to 100 mv. The resting potentials actually found ranged from 55 to 70 mv. Inspection of equation (17) reveals that it has two asymptotes intersecting at

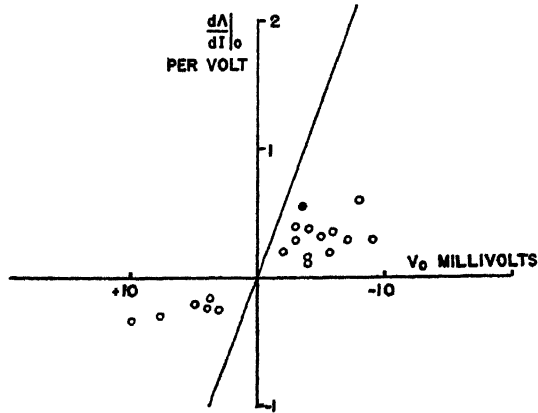


FIG. 10. Rate of change of conductance with current as a function of membrane potential for several artificial membranes and solutions. Open circles, concentration gradient of a single salt. Solid circle, two different chlorides at the same concentration. Two other such points could not be plotted as they fell outside the area of the diagram somewhat below the theoretical curve. Sign of potential as measured in external circuit.

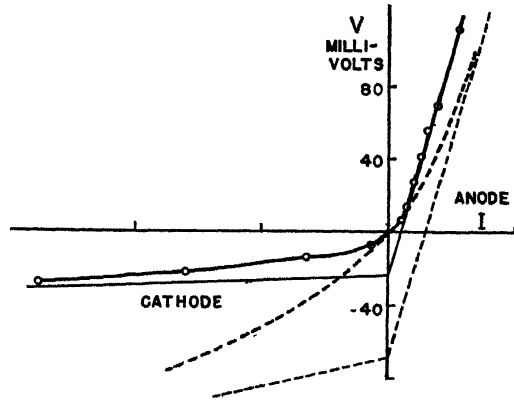


FIG. 11. Current-density *vs.* potential for squid axon. Circles and solid lines are experimental data. Dashed lines, theory for $V_0 = 70$ mv. The straight lines are the corresponding asymptotes. Since the actual membrane resistances are imperfectly known, the abscissa units are arbitrary.

$J = 0$ and $\Delta V = V_0$. In this way we obtain from the experimental curves 15 to 30 mv. Further, the ratio of limiting slopes may be used in equation (19) to get Δ_0 and here values are found 50 to 200 per cent of the actual ones. A curve is given in Fig. 11. It is thus evident that while the simple theory can account fairly well for the variation of resting potential with the external K^+ concentration, the axon is a much better rectifier than is predicted.

The rectification properties of the axon fade away as it dies or is narcotized (Guttman and Cole, 1941). This indicates either that the integrity of the membrane is affected or that the rectification depends on metabolic phenomena which supply a source or sink of K^+ . The present study appears, however, to strengthen the idea that the so called subthreshold electrical properties are interpretable in terms of a physical picture which does not involve metabolic activity. Cole and Marmont (1942) have shown that the impedance properties can vary considerably with the constitution of the environmental medium. As another possibility, Danielli (1941*a*) has suggested an interpretation of the "permeability" changes of nerve in terms of a bimolecular layer which could be profoundly altered by the enormous electric fields which might then be present.

The foregoing analysis has applied to systems for which a relatively simple structure might conceivably be postulated. In the presence of an asymmetrical membrane or of large charge asymmetries the complexities are much greater. Indeed, the more simple systems are themselves a severe strain on the analytical treatment and the rough correspondence observed may be fortuitous. The neglect of surface and body charges has been justified on the grounds of convenience, and while it is easy to show that external surface effects cannot contribute appreciably to the rectification, the internal charges cannot be so lightly dismissed. For example, insulating oils have been found to have a markedly non-linear field distribution (Whitehead and Minor, 1935) where the indifferent electrodes refused to allow the passage of ions and so caused a back diffusion into the body of the fluid. Labes (1932) has given a discussion similar to the Planck treatment for a membrane penetrated only by cations and has included surface charges and phase boundary potentials. The results do not appear to differ markedly from the simpler case.

Another approach is possible. If the material is dense enough, the ions may have to jump from point to point over potential barriers. This idea has been successfully applied to conduction in glass by Maurer (1941) and has been suggested by Danielli (1941*b*) for biological membranes. Whether such dense, rigid structures occur in any of the cases considered here is doubtful.

From the experimental point of view, the wide range of possibilities and the lack of uniform, stable material has both necessitated and interfered with the obtaining of a maximum of relevant data for each membrane. With biological systems this difficulty must be met in terms of the system studied. On the other hand, the present state of the technology of synthetic plastics encourages the belief that it should be possible to find materials for artificial membranes relatively free from this disadvantage. It is evident that materials are needed whose composition can be accurately stated and it would be particularly helpful to have data on membranes of a completely inert nature.

Diffusion studies have yielded much information about membrane properties. The analysis given here indicates that non-linearities are to be expected in

this case as well and that Fick's law may be no more valid than Ohm's law when applied to the entire membrane. This may have a bearing on some diffusion processes in biological systems.

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SUMMARY

Impedance and potential measurements have been made on a number of artificial membranes. Impedance changes were determined as functions of current and of the composition of the environmental solutions. It was shown that rectification is present in asymmetrical systems and that it increases with the membrane potential. The behavior in pairs of solutions of the same salt at different concentrations has formed the basis for the studies although a few experiments with different salts at the same concentrations gave results consistent with the conclusions drawn.

A theoretical picture has been presented based on the use of the general kinetic equations for ion motion under the influence of diffusion and electrical forces and on a consideration of possible membrane structures. The equations have been solved for two very simple cases; one based on the assumption of microscopic electroneutrality, and the other on the assumption of a constant electric field. The latter was found to give better results than the former in interpreting the data on potentials and rectification, showing agreement, however, of the right order of magnitude only. Although the indications are that a careful treatment of boundary conditions may result in better agreement with experiment, no attempt has been made to carry this through since the data now available are not sufficiently complete or reproducible. Applications of the second theoretical case to the squid giant axon have been made showing qualitative agreement with the rectification properties and very good agreement with the membrane potential data.

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NATURE OF THE ACTION CURRENT IN NITELLA

V. PARTIAL RESPONSE AND THE ALL-OR-NONE LAW

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PLATE 1

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Stimulation is normally followed by a process of recovery which restores the cell to the state which existed before stimulation. If a new stimulus arrives before recovery is complete there may be no response or only a partial one.

This can be best understood if we begin by a brief discussion of the structure of the protoplasm.

Experiments indicate that the protoplasm consists of an aqueous layer *W* bounded by an outer non-aqueous layer *X* and an inner non-aqueous layer *Y*. Under normal conditions there is an outwardly directed concentration gradient¹ of KCl across *Y* and a very much smaller one across *X*: hence we find a large outwardly directed potential at *Y* and a much smaller one at *X*.²

Stimulation appears to be associated with an increase of the permeability³ of *Y* and an outward movement of K^+ resulting in a loss of the concentration gradient, and consequently of the potential, across *Y*. This causes the sudden rise which constitutes the spike of the action curve (*a*, Text-fig. 1).

When the outwardly moving K^+ reaches⁴ *X* it may set up a sufficient concentration gradient to cause an outwardly directed potential at *X* (*b*, Text-

¹ For convenience we speak only of KCl since it is the most important substance in this connection, but other potassium salts act like KCl and to a lesser degree salts, of sodium. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215; 1939-40s **23**, 171.

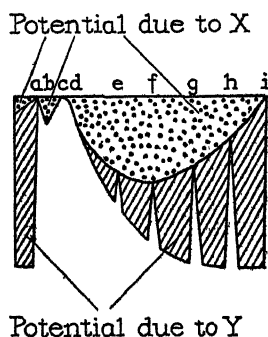
² This becomes evident when the potential due to *X* is removed by leaching with distilled water (Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) which makes *X* insensitive to K^+ . This does not appear to diminish the total potential.

³ Stimulation is accompanied by a great increase in electrical conductivity. It is significant that the exit of KCl (which probably occurs) would assist this since we find that placing KCl on the outside lowers resistance. Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 495; 1936-37, **20**, 229. Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938-39, **22**, 37.

⁴ With *Y* very permeable the time required for this appears to be not more than 1 second since the minimum distance between *Y* and *X* (i.e., between the chloroplasts) is considerably less than 10 microns. When tap water saturated with chloroform is applied externally *X* is made permeable and the chloroform may penetrate to *Y* in less than 2 seconds.

fig. 1). This disappears as K^+ passes out⁵ through X and thus destroys the concentration gradient across X ; in consequence we may find (as explained in previous papers⁶) a second peak in the action curve (c, Text-fig. 1).

The action curve now begins to descend as the process of recovery sets in. This involves the return⁷ of the cell to its original state and consequently the inward movement of K^+ from W through Y into the sap. As this continues the outwardly directed concentration gradient of K^+ across Y (and conse-



TEXT-FIG. 1. Hypothetical diagram of the distribution of potential in the protoplasm which is supposed to consist of an aqueous portion W bounded by two very thin non-aqueous layers (X , at the outer surface, and Y , at the inner surface).

When stimulation occurs Y loses its potential and this produces the sudden rise (spike) of the action curve at a . Potassium moves outward and on reaching X sets up some potential (b) which disappears when the potassium reaches the outside of X (at c).

The process of recovery now sets in and potassium moves back into the sap, decreasing the potassium outside of X and increasing it inside of X and thus increasing the potential across X . As potassium continues to move inward its concentration just inside X decreases and the potential across X decreases. Hence the potential due to X first increases and then decreases.

When stimulation occurs during recovery Y loses its potential but X does not: hence there is no response at d , and the responses at e , f , g , and h are incomplete but they increase as recovery proceeds because the potential at Y increases.

quently the potential across Y) increases and the action curve descends toward the base line from which it started.

⁵ The rapidity of this outward movement of K^+ will depend on the permeability of X .

⁶ Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1939-40, **23**, 743; 1939-41, **24**, 9.

⁷ This includes the return of the cell to its normal resistance and to its usual behavior in respect to apparent capacity. Blinks, L. R., *J. Gen. Physiol.*, 1936-37, **20**, 229. Cole, K. S., and Curtis, H. J., *J. Gen. Physiol.*, 1938-39, **22**, 37.

This inward movement of K^+ at first diminishes the concentration of K^+ outside of X and increases its concentration just inside of X ⁸ and hence increases the outwardly directed concentration gradient of K^+ across X and the potential across X . But as this process continues and K^+ moves inward, causing the concentration just inside X to fall off, the concentration gradient—and consequently the potential—across X falls off. Hence the potential across X first increases and then decreases as shown in Text-fig. 1.

Let us now consider what happens if stimulation occurs during the process of recovery. We suppose that Y loses its potential as usual and K^+ comes out of the sap into W . If an impulse arrives at d , Text-fig. 1, there will be no apparent response because Y has no potential to lose. If it arrives at e the action curve will be small because Y has very little potential to lose. Since W now has a concentration of K^+ much greater than usual there will be very little outward movement of K^+ and this can be quickly reversed in the recovery which sets in immediately afterwards. Little or no effect on the potential across X can be expected because there will be little or no diffusion of K^+ to X and any diffusion boundary will not be sharp. The net result will be a small rise in the action curve with a quick return to the base line (e , Text-fig. 1).

A stimulus occurring a little later finds Y with more potential on hand (f , Text-fig. 1) and consequently the loss of this potential causes the action curve to rise more. Subsequent stimulations find Y with more and more potential and therefore the action curve rises higher and higher until it reaches the magnitude it had at the start. We assume that stimulation causes no loss of potential at X .⁹

It is evident that an impulse arriving before recovery is complete can produce only a partial response; *i.e.*, one that is less than normal in magnitude. Even when the action curve has descended to the base line recovery may not be complete since part of the potential may be due to X . As stimulation does not cause a loss of this potential but only of the potential due to Y the response is only a partial one¹⁰ and does not go to zero.

Fig. 1 shows a photographic record which resembles Text-fig. 1. The

⁸ This is because K^+ does not pass inward by simple diffusion but by a process of accumulation (requiring an expenditure of energy). This process a little later causes the concentration of K^+ in the sap to exceed that in W . It is a process which tends to go on continuously in the cell unless interrupted by stimulation which causes K^+ to move out through Y and in some cases through X . Cf. Osterhout, W. J. V., *Bot. Rev.*, 1936, 2, 283.

⁹ Experiments have shown that the loss of potential at Y does not involve loss of potential at X . Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 420 (Fig. 3).

¹⁰ To what extent this explanation applies to muscle and nerve remains an open question. Regarding the possibility of a double surface in muscle see Francis, W. L., *Proc. Roy. Soc. London, Series B*, 1937, 122, 140.

record is made from a cell in which two places, lettered from left to right *C* and *D*, were connected to a spot *F* still further to the right, as described in former papers.¹¹

The cell was kept for 1 hour in 0.01 M NaCl¹² before the record was made. During this exposure the time required for full recovery was reduced from about 20 seconds to about 0.7 second and a pacemaker became established somewhere to the left of *C*. From this impulses passed to *C* and *D*, eliciting full responses at each, so that a long train of action curves appeared at the rate of 7 in each 5 seconds (these responses were practically identical with the full responses seen in the record at *C* in Figs. 1 and 2).

Then at *D* 0.01 M NaCl was replaced by 0.001 M NaCl + 0.0005 M CaCl₂ accompanied by some manipulation of the cell at *C*. This changed the pattern at *C* to that seen in Fig. 1. This is a variation of electrical alternans as described in a former paper¹³ and consists of a full response followed as a rule by two smaller responses.

At *D* the time required for full recovery was lengthened from 0.7 to about 13 seconds thus making it possible for impulses from *C* to arrive at *D* before *D* had recovered from a previous stimulation.

In Fig. 1 we see that the third full response at *C* is not followed by a response at *D*. This may mean that the impulses coming from *C* arrived while *D* was in the absolutely refractory state and *Y* was unable to respond. But even if *Y* did respond its loss of potential might be too small to be detected (the total potential was small and it may have been all located at *X*).

The next full response at *C* is followed by a small response at *D*. As recovery at *D* progresses the successive responses become greater.

Although there is essential agreement between Text-fig. 1 and Fig. 1 there are some minor points of difference. In the first full response at *D* in Fig. 1 the curve descends sharply after the spike, presumably because K⁺ reaches *X* and builds up a potential there. The curve then begins to rise because K⁺ passes

¹¹ The cells, after being freed from neighboring cells, stood in the laboratory at 15°C. ± 1°C. in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) for several days. They belonged to Lot B (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, 24, 312).

The measurements were made on *Nitella flexilis*, Ag., using the technique described in former papers (Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, 21, 541). Temperature 20-26°C. Regarding the amplifier see the reference just cited.

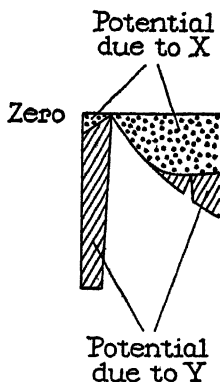
There was no indication of injury in these experiments.

¹² The effect of NaCl may be due to increased conductivity of the aqueous layer of the protoplasm *W* associated with an increased tendency of *Y* to become permeable more easily as the result of stimulation. Cf. Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 91.

¹³ Osterhout, W. J. V., *J. Gen. Physiol.*, 1942-43, 26, 457.

out through X and thus lessens the concentration gradient of K^+ across X . If this process goes far enough the curve rises to zero as in Text-fig. 1 but if recovery sets in before this happens the second peak does not reach zero: this is the state of affairs in Fig. 1.

The amount of K^+ passing out depends on the permeability of X and on the steepness of the concentration gradient of K^+ as it approaches X , *i.e.*, on the sharpness of the outwardly moving diffusion boundary of K^+ . If K^+ does not pass out through X rapidly enough to lower the concentration gradient across X to a sufficient extent before recovery (involving the inward movement of K^+) sets in¹⁴ there will be no double peak (Text-fig. 2). It is therefore not surprising that we see single peaks in Figs. 1 and 2.



TEXT-FIG. 2. Hypothetical diagram to explain the occurrence of a single peak. We suppose that K^+ moving outward comes in contact with X and sets up a potential but very little K^+ passes out through X before recovery sets in, involving the inward movement of K^+ and the eventual loss of potential due to X (this loss is not shown in the diagram).

The quick onset of potential due to X produces a quick downward movement and a sharp peak as in Text-fig. 2, but it is of interest to note that we find, as expected, a rounded one in *Chara* where there is no potential due to X (because X is insensitive to K^+).¹⁵ Here the downward movement depends solely on the

¹⁴ In this case the potential due to X increases as K^+ moves out of the sap into W , *i.e.*, at a time when the potential due to Y is not increasing. But in Fig. 1 the potential due to X increases as K^+ moves inward; *i.e.*, as the potential due to Y is increasing. Hence with a double peak we may expect to see the two potentials increasing together to some extent but not so with a single peak. This corresponds to observation.

¹⁵ Osterhout, W. J. V., *J. Gen. Physiol.*, 1934-35, **18**, 215; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1940-41, **24**, 9.

inward movement of K^+ into the sap and the consequent increase in the potential due to Y . This is relatively slow and hence gives a rounded peak.

In Fig. 2 the partial responses increase in magnitude and then decrease. This is not surprising as the decrease begins at a time when the concentration of K^+ in W and the potential across X are falling off (*cf.* Text-fig. 1). At this time, therefore, K^+ coming out of the sap into W as the result of stimulation causes greater changes in the concentration of K^+ in W and may consequently produce greater changes in potential. Since the amount of K^+ coming out as well as the concentration already present in W would depend on a variety of conditions there is evidently considerable opportunity for variation in the action curves. This will receive attention in subsequent papers.

When there is no spontaneous activity we may employ electrical stimulation, as in Fig. 3, where the recovery time is longer and the resulting pattern is irregular.

In many cases where the cell has not been treated with NaCl and the recovery time is relatively long no partial responses are seen. The cell refuses to respond at all until recovery is complete and it then gives a full response.

Let us now consider the behavior of C (Figs. 1 and 2) which shows some interesting features. In the earlier part of the record (not shown here) before the cell was manipulated at C all the responses were full responses at the rate of 7 in 5 seconds. After manipulation the pattern at C changed to that seen in Fig. 1, *i.e.* a full response followed usually by 2 partial responses,¹⁶ the latter being preceded by a positive dip in the curve. This positive dip is also seen at D where the 2 subsequent partial responses can be made out in some cases.

We may ask why the partial responses at C are so small. If the potential at Y is very small when they occur we may suppose that this potential is wholly lost on the all-or-none principle. Otherwise we might assume that the all-or-none law is not obeyed and that only part of the potential is lost. This might conceivably be caused by changes in ionic mobilities or in partition coefficients in X or Y . Such alterations have been observed as the result of metabolic¹⁷ changes and of the application of reagents¹⁸ and might conceivably be produced by stimulation. In some cases where the partial response is very small such an explanation may be indicated. Otherwise we should have to assume improbable values of the potential due to X especially when there are large fluctuations in this value as measured by the distance between zero and the apex of the partial response.

¹⁶ This is a variation of electrical alternans. For somewhat similar patterns see Osterhout, W. J. V., *J. Gen. Physiol.*, 1942-43, **26**, 457, Fig. 3.

¹⁷ Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 429.

¹⁸ Osterhout, W. J. V., *J. Gen. Physiol.*, 1936-37, **20**, 13, 685; 1937-38, **21**, 707; 1938-39, **22**, 417; 1939-40, **23**, 171, 569, 749; 1940-41, **24**, 699. *J. Cell. and Comp. Physiol.*, 1941, **18**, 129.

Another possibility is suggested by the work of Hodgkin,¹⁹ who finds that an electrical disturbance in nerve may produce an electrical change beyond a block without necessarily involving any physiological response at the spot beyond the block. If the two small movements coming after each spike at *C* involve no physiological response and the similar movements at *D* are of the same sort the movements at *D* should be simultaneous with those at *C* and of smaller magnitude.²⁰ Whether they are simultaneous is not clear but their magnitude appears to be less and to fall off still more as the impulse travels further along the cell, as is evident in another part of the record not shown here.

SUMMARY

When a stimulus arrives before recovery is complete there may be no response or only a partial response. A typical response appears to involve an immediate loss of potential at the inner protoplasmic surface but not at the outer surface. As long as recovery is incomplete only a part of the total potential is located at the inner protoplasmic surface and the loss of this part of the total potential can cause only a partial response; *i.e.*, one of smaller magnitude than the normal.

Even after the action curve has returned to the base line recovery may be incomplete and the response only a partial one. The return of the action curve to the base line means a recovery of total potential but if part of this is located at the outer protoplasmic surface and if this part is not lost when stimulation occurs the response can be only a partial one. During recovery there is a shift of potential from the outer to the inner protoplasmic surface. Not until this shift is completed can recovery be called complete. The response to stimulation then becomes normal because the loss of potential reaches the normal amount.

In many cases the partial responses appear to conform to the all-or-none law. In other cases this is doubtful.

¹⁹ Hodgkin, A. L., *J. Physiol.*, 1937, **90**, 183.

²⁰ The distance between *C* and *D* is 1 cm.

EXPLANATION OF PLATE 1

FIG. 1. Shows the effect of stimulation before recovery is complete.

Two places on the cell lettered from left to right *C* and *D* are connected to a spot *F* still further to the right. The cell was left in 0.01 M NaCl for 1 hour until a pace-maker was established to the left of *C* which sent impulses along the cell to *C* and to *D* (there was no electrical stimulation). *F* was in contact with 0.01 M KCl which kept the P.D. constant approximately at zero.

At *C* we find as a rule a full response going approximately to zero followed by 2 small partial responses.¹⁶

While *C* was left in contact with 0.01 M NaCl *D* was placed in contact with 0.005 M NaCl + 0.0025 M CaCl₂ which lengthened the time of recovery at *D* so that some of the impulses coming from *C* were unable to elicit a full response at *D* because they arrived before recovery was complete.

The third full response at *C* is not followed by a response at *D*: after this, responses of increasing magnitude are observed at *D* until the response becomes nearly complete and a new series begins.

It should be noted that even after the action curve has descended to the base line from which it started recovery is incomplete and the response is incomplete. For explanation see Text-fig. 1.

The cell was freed from neighboring cells and kept in Solution A at 15°C. \pm 1°C. for 30 days and then placed in 0.01 M NaCl for 1 hour at about 22°C. before the record was made.

Heavy time marks 5 seconds apart.

FIG. 2. Later section of the record shown in Fig. 1. The partial responses at *D* first increase and then decrease.

FIG. 3. Shows the effect of electrical stimulation before recovery is complete: each electrical stimulus is indicated by a signal above the action curve (these signals are about 10 seconds apart). The portion of the photographic print showing the signals was cut out and pasted on: it has not shrunk evenly in drying and in consequence the lines do not all match exactly.

The stimulating electrode was 2 cm. from the recorded spot and no leakage effect (shock artifact) is evident.

The spot recorded was in contact with 0.01 M NaCl and was connected to a spot in contact with 0.01 M KCl which kept the P.D. constant approximately at zero.

The cell was freed from neighboring cells and kept in Solution A at 15°C. \pm 1°C. for 9 days. It was then placed for 1 hour in 0.01 M NaCl at about 22°C. before the record was made.

Time marks 5 seconds apart.

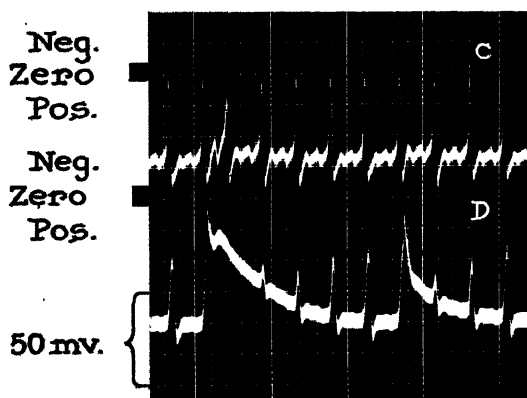


FIG. 1

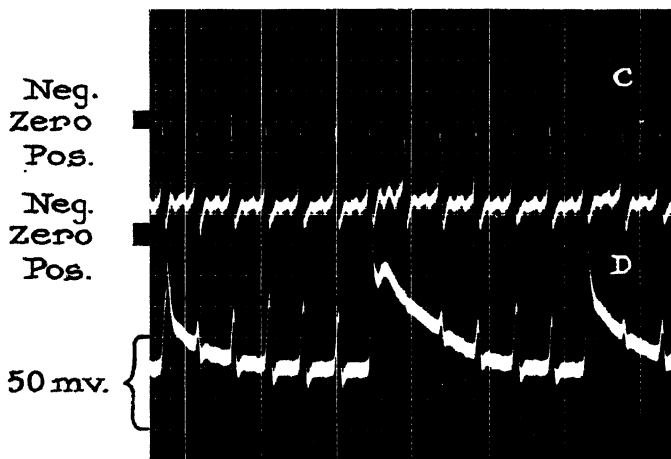


FIG. 2

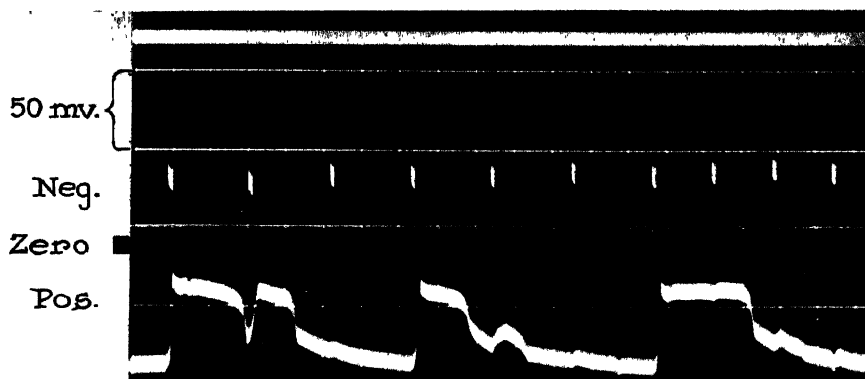


FIG. 3

THE QUANTITATIVE EFFECT OF X-RAYS ON ASCORBIC ACID IN SIMPLE SOLUTION AND IN MIXTURES OF NATURALLY OCCURRING COMPOUNDS*†

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Among the many studies that have been made on biological radiation effects, relatively few (1) have given attention to the question of what compounds in tissue may react when it is irradiated. This report is concerned with a part of an approach to this question through the study of competitive reactions in aqueous solutions.

Several facts suggest the desirability of such an approach. First, in soft tissue irradiated by high voltage x-rays the bulk of the energy absorbed will be absorbed in the water and the next largest amount in proteins as a class.¹ Most of the energy available for causing immediate reaction is therefore in many tissues initially in the water, yet even there the primary chemical reactions produced are quantitatively very small. As is plausible from purely physical data, Fricke (2) and others (3-5) working in dilute aqueous solutions have usually found that 1000 r produced measurable changes in concentration of at most a few micromoles per liter. This amount of radiation may produce profound biological effects. Finally, a great variety of inorganic and organic compounds will react in dilute aqueous solutions under the influence of x-rays.² While these reactions with ionized water³ are, of course, not identical they are certainly not all independent of each other and many must occur with the same x-ray products. For instance, it is now believed, following the original suggestion by Fricke (6), that a variety of proteins, including enzymes, papilloma virus (7-8), and bacteriophage (9), compete in part with other proteins and other compounds (10) for these products. In a mixture such as non-bony

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† Read at the 105th Meeting of the American Chemical Society, Detroit, Michigan, April 12 to 16, 1943.

¹ This characteristic is, of course, in marked contrast to that of the usual ultra-violet, 2500Å or more.

² An extensive list of references to studies on irradiated water solutions is given by Fricke (2).

³ The phrase "ionized water" is intended to include any reactive entity which results immediately from energy absorption in the water and which involves only water and oxygen.

tissue, therefore, the many compounds there which are capable of reacting will, to an unknown but surely large extent, compete⁴ for the limited amount of ionized water.

In the past, the mere fact of reaction in dilute aqueous solution has at times been adduced as evidence for the reaction of a particular compound in tissue. It is clear that while an ability to react in pure solution may be necessary, it alone gives no clue regarding the result in a complicated system. However, from a study of relatively simple mixtures, it should be possible to learn whether compounds exist which are capable of competing successfully for far more than their proportionate share of the total available ionized water.

In the initial phase of this work (11) a study was made of the x-ray inactivation of luciferin in aqueous solutions containing one of a series of compounds, including ascorbic acid. The loss of luciferin was less when the irradiated solution contained ascorbic acid than when it did not. This suggested the desirability of studying the x-ray induced reaction of ascorbic acid itself to see whether or not this reaction continued in the presence of other compounds, particularly the proteins. The results of these experiments are given below.

Methods

Stock solutions of ascorbic acid were prepared by dissolving 0.1 gm. of Merck's crystalline material in 100 ml. of 5 per cent metaphosphoric acid. Such solutions were used as the standard. Solutions to be irradiated were prepared by diluting the stock solution and neutralizing it slowly with the proper amount of sodium hydroxide in the presence of phosphate buffer of pH 6.8. The final concentration of buffer was 0.025 M. Determinations of the ascorbic acid content of control solutions were made at the beginning and end of the experiments, since spontaneous oxidation occurred in some cases. However, by chilling the solutions in an ice bath and keeping the time as short as possible the loss was not usually greater than the errors in the determination.

The titrations of ascorbic acid were made with Eastman's sodium 2,6-dichlorobenzenone indophenol (12-13) in solutions acidified with $\frac{1}{2}$ or $\frac{1}{3}$ the volume of 5 per cent metaphosphoric acid. Where proteins were present, enough 5 per cent metaphosphoric acid was added to insure complete precipitation, usually $1\frac{1}{2}$ volumes, and the precipitate removed by centrifuging before titration.

Serum albumin was prepared from dried human plasma by the method of Svcdberg and Sjögren (14) except that it was not reprecipitated.

Irradiation was carried out with unfiltered x-rays produced at 185 kv. The intensity was about 5000 r per minute measured in air. Exposure of solutions was in pyrex test tubes, the walls of which did not reduce the intensity of radiation by more than 10 per cent. The amounts of radiation quoted are therefore all based on the intensity in air.

⁴ Examples are known (5) where some enhancement of the radiation effect on a given compound occurs in the presence of other compounds but these are small in comparison to the inhibitions, indicative of competition, that have been observed.

Distilled water was either from a tin still or redistilled from pyrex glass. For the comparative purposes of this investigation, it did not seem necessary to take the additional precautions used by Fricke (2).

RESULTS

The data in Table I show the extent to which ascorbic acid in a solution containing only inorganic constituents, 0.025 M phosphate buffer and about 0.5 M sodium metaphosphate, reacts under the influence of x-rays. Very low concentrations of ascorbic acid were used in order to increase the relative size of the x-ray effect with moderate amounts of radiation. About one-half of the ascorbic acid reacted upon exposure to 5500 r. The data show a reasonable reproducibility in a series of comparable experiments. They also show that under

TABLE I

Concentration of unirradiated ascorbic acid solutions	Loss of concentration after		Loss per 1000 r	
	5,500 r	11,000 r		
<i>mg./100 ml.</i>	<i>mg./100 ml.</i>	<i>mg./100 ml.</i>	<i>mg./100 ml.</i>	<i>μ moles/1000 ml.</i>
0.36		0.32	0.029	1.7
0.43		0.35	0.032	1.8
0.45	0.23		0.042	2.4
0.43	0.22		0.040	2.3
0.42	0.21		0.038	2.2

The concentration of each unirradiated ascorbic acid solution is the average of the concentrations found at the beginning and end of the experiment. Each value given as a loss in concentration is the average value found in two to four solutions irradiated at the same time.

these conditions 1000 r produced changes in concentration of about 2 micromoles per liter. This is definitely not an absolute value since it apparently is dependent on the extent of the reaction and depends on other variables such as the absolute concentration and the pH. That the bulk of the reaction occurs during the irradiation, or at most a very few minutes thereafter, was shown by titrating at intervals a series of solutions irradiated at the same time. The average values for the remaining ascorbic found in the titrations done first, that is within a few minutes after the end of the irradiation, and the last, done perhaps half an hour later, were 0.23 and 0.20 mg./100 ml. The difference is within the error.

The extent to which ascorbic acid reacts under a particular set of conditions in relatively simple solutions having been shown, the next point to be studied was what part, if any, of this reaction would occur in the presence of proteins. To test this, the data given in Table II were obtained. It is evident that 0.2 per cent serum albumin, about 5×10^{-5} M, does not reduce the radiation effect

on 3.5×10^{-6} M ascorbic acid by more than the variation between the individual control experiments.

Since a single experiment with 2.5 per cent albumin still showed the usual loss of the ascorbic acid, the behavior of the reaction in a much more complicated system, blood plasma, was tested. Dried plasma, to which ascorbic acid had been added, was first used, followed by a few confirmatory experiments with fresh plasma. These results are given in Table III. For 11,000 r, the average

TABLE II
Loss of Ascorbic Acid

Concentrations of the unirradiated solutions are given at the head of each column.

Amount of irradiation					0.2 per cent serum albumin		
	0.57	0.53	0.51	Average	0.60	0.62	Average
0	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.
2750r	0.11	0.12	0.14	0.12	0.08	0.11	0.10
5500r	0.22	0.20	0.22	0.21	0.19	0.20	0.20
11,000r	0.36	0.33	0.37	0.35	0.35	0.38	0.37
16,500r	0.46	0.42	0.45	0.44	0.50	0.51	0.51

TABLE III

Ascorbic acid irradiated in	Amount of irradiation	Concentration of ascorbic acid				Average loss
		mg./100 ml.	mg./100 ml.	mg./100 ml.	mg./100 ml.	
Buffer solution	0	0.41	0.40	0.43	0.43	
	11,000	0.10	0.07	0.06	0.08	0.34
7 per cent dried plasma	0	0.47	0.46	0.47	0.42	
	11,000	0.17	0.17	0.18	0.16	0.29
Fresh plasma	0	0.57	0.83	0.86		
	5,500	0.39				0.18
	11,000	0.25				0.32
	22,000		0.16	0.19		0.67

loss from the controls is 0.34 mg./100 ml., while from the dried plasma it is 0.29 mg./100 ml. The data show that the ascorbic acid reaction continues even in fresh plasma.

It was therefore of interest to know what happens to the reaction in a still more complicated system such as muscle. A large number of experiments were carried out in which muscle tissue was irradiated after removal from the animal. Table IV shows experiments on muscle taken from the hind legs of rats. The muscles were cut into pieces with scissors, either immediately or 24 hours after removal from the animal, the pieces mixed thoroughly, and then a number of samples were taken as controls and for irradiation. The data shown

TABLE IV
Ascorbic Acid in Milligrams per 100 Gm. of Rat Muscle

Experiment	Controls	After 22,000 r	Differences
	<i>mg./100 gm.</i>	<i>mg./100 gm.</i>	<i>mg./100 gm.</i>
108	2.73 2.47	2.67 2.17	-0.18
112	1.15 1.29	0.87 0.73	-0.42
116	2.53 2.30 2.25	2.12 1.96	-0.32
117	1.70 1.74	1.80 1.91	+0.14
118	1.30 1.10	1.26 1.12	-0.01
119	1.76 1.82	1.76 1.78	-0.02
121	1.82 1.64 1.69	1.42 1.48 1.51	-0.25
122	1.92 1.66 1.65	0.99 1.29 1.38	-0.52
123	1.76 1.75 1.66	1.33 1.47 1.44	-0.31
127	1.41 1.42	1.41 1.42	-0.00
128	1.60 1.57	1.31 1.54	-0.16
Average.....			-0.19

are from only those experiments in which the controls showed reasonable agreement. The average difference between the controls and the samples given 22,000 r of irradiation is only 0.2 mg./100 gm., compared with nearly 0.7 mg./100 ml., in blood plasma. If two out of the 25 pairs of samples are

omitted, the average difference between control and irradiated muscle becomes only 0.15 mg./100 gm. The questions of whether or not this average apparent effect is real and whether the variability is due to random error or to real differences in the tissue need not be considered now. The important point shown in this series of experiments is that in most of the excised rat muscle tissue, the observed effect of x-rays on ascorbic acid is relatively small.

DISCUSSION

The change produced in ascorbic acid per 1000 r is within the usual range of 1 to 4 micromoles per liter reported for a number of organic compounds (2, 4, 15) under various conditions. It is reasonable to assume, therefore, that the ascorbic acid reaction represents a substantial fraction of the total ionized or activated water available. Hence it is of considerable interest that the change occurs without great interference in the presence of all of the compounds in oxalated blood plasma at the concentrations in which they are there found. This is particularly striking in the case of the albumins which, even on a molar basis, were 15 or 20 times as concentrated as the ascorbic acid. This is in contrast to the behavior of the x-ray induced reactions of ferrous sulfate and of thiamin. In unpublished work⁵ it has been found that both reactions are hindered by the presence of a few tenths per cent of serum proteins. Whether or not this property of ascorbic acid is due solely to an exceptional speed of reaction with ionized water is, of course, not known. Possibly it reacts secondarily with other affected compounds since it is such a good reducing agent. In any case, it seems unlikely that ascorbic acid is the only substance capable of reacting to an unusual extent in the presence of other naturally occurring compounds. Not all of the reactive entities formed in water by radiation would be expected to react with ascorbic acid. Also, the difference in the behavior of the reaction in plasma and in muscle suggests that in the latter, compounds are present which do interfere with the reaction of ascorbic acid. This may be due to compounds absent from plasma or present there in much lower concentration. Work is now in progress on this phase of the problem.

There is no suggestion from this work that small losses of ascorbic acid which may occur are, as such, factors in the destructive biological effects of x-rays. However, in view of its very wide distribution, the possibility that ascorbic acid exerts significant protective action on important compounds such as the proteins, or in biological effects, is being investigated. It would also be of interest to know the competitive behavior in simple solutions of ascorbic acid, and other compounds, with auxin because of Skoog's report (1) on the relation of auxin to some radiation effects on plants.

⁵ These experiments were carried out by Mr. Bieber.

SUMMARY

Data on the x-ray induced reaction of ascorbic acid in simple inorganic solution, in solutions containing serum albumin, in plasma, and in muscle have been presented. The reaction occurred in the presence of serum albumin and in human plasma but was relatively small in excised rat muscle.

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THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

VII. WATER UPTAKE AND SWELLING OF COLLODION MEMBRANES IN WATER AND SOLUTIONS OF STRONG INORGANIC ELECTROLYTES

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I

In preceding papers in this series we have dealt on the one hand with the chemical factors which determine the behavior of collodion membranes in solutions of strong electrolytes,¹⁻³ and on the other hand^{4, 5} we have shown that the collodion membrane does not behave as a homogeneous interphase but that it has a micellar structure.

To get a clearer picture of the structure of the collodion membrane it is necessary to know if it is permanent and rigid, or if it undergoes changes when it is immersed in different solutions. From our previous knowledge no general answer can be given to this question. Concentrated solutions of many organic compounds are known to swell collodion membranes appreciably. Water and aqueous solutions of inorganic compounds, however, are generally believed to exert no specific influence on collodion membranes, but this has never been proven definitely. The present investigation is concerned with the changes in collodion membranes produced by water and aqueous solutions of strong inorganic electrolytes; the more complex phenomena occurring in aqueous solutions of organic compounds will be taken up in a subsequent communication.

There are two general types of collodion membranes:

(a) "Porous" collodion membranes are obtained by casting collodion films from ether-alcoholic or similar solutions and immersing them in water before the solvent has evaporated completely. The porosity of these membranes depends on the content of organic solvent at the time of their immersion in water; to retain this porosity the membranes must be stored continuously in water.

(b) "Dried" collodion membranes are obtained if the solvent is allowed to evaporate completely from a film of collodion, or if "porous" membranes are

¹ Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

² Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

³ Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, **25**, 411.

⁴ Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1942, **26**, 17.

⁵ Sollner, K., and Carr, C. W., *J. Gen. Physiol.*, 1943, **26**, 309.

allowed to dry completely in air. When such membranes are placed in water, they take up a small percentage of water but do not swell to form a porous structure of the type of the "porous" membranes. Membranes in this state will be called "water-wetted dried" membranes. They have been shown to be of porous character;^{4, 5} *i.e.*, they contain pores, but the porosity is at a minimum.

In the past there has been some question concerning the way in which the water enters a dried membrane. The problem arises as to whether the water dissolves in the collodion or whether it enters pores as in porous clay membranes. In either case the assumption has always been made that the membrane does not swell, but this has not been proven.

Northrop⁶ has shown that the amount of water retained by dried collodion is directly proportional to the water vapor pressure at the time of drying. Thus in a completely dry atmosphere all of the water can be removed. From these experiments Northrop postulates that the water goes into solution in the collodion. If this were the case, one would expect the membrane to swell to a certain extent; however, Northrop has not followed up this point.

Michaelis in his studies on the dried collodion membrane comes to the conclusion that it behaves as a rigid, porous structure which shows no trace of swelling after wetting, and that water enters the membrane by filling preformed pores.^{7, 8} These preformed pores would be the micellar interstices which are assumed to exist between the collodion micelles forming the membrane. It was not shown, however, that the membrane had exactly the same structure when completely dry as when wetted with water.

Another investigator of collodion membranes, Manegold,⁹ states specifically that dried collodion is non-swelling in water, but he gives no experimental proof of this statement.

The only information in the literature on measurements of volume changes in membranes pertains to membranes other than collodion. Zsigmondy membrane filters,¹⁰ which are acetyl cellulose membranes,¹¹ have been found by Brukner¹² to shrink slightly in high concentrations of electrolytes. In 1 M NaCl he found a shrinkage of about 0.4 per cent in length and width of these membranes, and in 4 M NaCl the shrinkage went up to 1 per cent. McBain and

⁶ Northrop, J. H., *J. Gen. Physiol.*, 1928, **12**, 435.

⁷ Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1928, **12**, 221.

⁸ Michaelis, L., *Bull. Nat. Research Council*, No. 69, 1929.

⁹ Manegold, E., *Kolloid-Z.*, 1932, **61**, 140.

¹⁰ Zsigmondy, R., and Bachmann, W., *Z. anorgan. u. allg. Chem.*, 1918, **103**, 1.

¹¹ Bachmann, W., *Kolloidchemische Untersuchungsmethode*, in Berl-Lunge, *Chemische-technische Untersuchungsmethoden*, Berlin, Julius Springer, 8th edition, 1934, **1**, 1087.

¹² Brukner, Z. *Ver. deutsch. Zuckerind.*, 1926, **76**, 3. (Quoted by Manegold, E., and Hoffmann, R., *Kolloid-Z.*, 1930, **50**, 207.)

Stuewer¹³ measured the swelling of dry cellophane sheets when placed in water. This was done by determining the changes in each of the three dimensions, and it was found that the cellophane increased more than 100 per cent in thickness, about 10 per cent in width, but did not change in length. In addition, these authors have shown that the swelling is even more pronounced in strong solutions of ZnCl_2 . Membranes previously swelled in water were found to double their thickness again when placed in 65 per cent ZnCl_2 solution.

The current opinion¹⁴ concerning the swelling of cellulose in water is that the unsubstituted hydroxyl groups in cellulose (cellophane) are responsible for its swelling. One should remember here that collodion is not a completely nitrated cellulose, for cellulose hexanitrate contains 14.14 per cent nitrogen as compared with 10.0–11.5 per cent nitrogen for collodion. Thus collodion has some unsubstituted hydroxyl groups, and these might easily determine its behavior in water.

In the experimental parts of this paper the following questions will be answered: (1) How much water does a completely dried collodion membrane take up when placed in water? (2) Does this water uptake cause any detectable change in the volume of the membrane? (3) Do dried collodion membranes swell or shrink when transferred from water to solutions of strong electrolytes? (4) Do "porous" collodion membranes swell or shrink when transferred from water to solutions of strong electrolytes?

II

For the determination of the water uptake and possible volume changes of dried membranes, flat membranes similar to those used by most investigators proved to be the most suitable. Such membranes were completely dehydrated and then their weights and volumes were measured. After being immersed in water for some time, the membranes were again measured for their weight and volume. The increase in weight is equal to the water uptake, and any change in volume indicates swelling or shrinking.

The dried membranes were prepared as follows: 150 to 200 ml. of a 5 per cent collodion solution in ether-alcohol was poured out onto six perfectly flat glass plates ($3\frac{1}{4}'' \times 4''$ photographic plates) which floated on and nearly covered a mercury surface. In casting the membranes the formation of gas bubbles was carefully avoided. After the collodion had dried in air for 24 hours, it was covered with water and allowed to stand for 4 to 5 hours. Then the membrane can be loosened easily from the glass plates. A large sheet of dried collodion was thus obtained, on which six rectangles were outlined by the glass plates. These were then cut into 3×4 cm. membranes which were kept for further use in a desiccator. The thickness of these membranes in different castings varied between 0.10 and 0.15 mm.

¹³ McBain, J. W., and Stuewer, R. F., *J. Physic. Chem.*, 1936, **40**, 1157.

¹⁴ Gortner, R. A., *Outlines of biochemistry*, New York, John Wiley and Sons, 2nd edition, 1938, 682.

The volume change of dried collodion when immersed in water was determined with a pycnometer filled with mercury. Mercury was used as the reference liquid for two reasons. In the first place it was necessary to use mercury because it does not wet collodion, *i.e.* it does not enter any preformed pores that might exist, and it does not make the collodion swell. Water or any other liquid that wet collodion would obviously not give the true volume of a dried membrane, for in such a liquid it would no longer be in the dry state. In addition the use of mercury was advantageous since because of the great difference in density of collodion and mercury, small changes in volume could easily be detected.

The pycnometer was a 10 ml. glass bottle with a ground glass stopper. There was a small hole in the center of the stopper, and this extended into a capillary tubing of 1 mm. bore and 200 mm. in length. The pycnometer was first filled with mercury, an extra drop being left at the top of the capillary, and then clamped in a constant temperature bath ($\pm 0.05^\circ\text{C}.$). After temperature equilibrium was reached (less than 5 minutes) the drop at the top of the capillary was cut off with a knife-edge. This procedure was necessary because a change of 1° in temperature changed the total weight by 10 mg. The pycnometer then was taken out of the bath, wiped dry with filter paper, and weighed to within 1 mg. By repeating this procedure it was possible to make a number of measurements that agreed within 3 mg., the total weight being approximately 190 gm.

To make the weight and volume determinations, a dried membrane was first weighed to within 0.3 mg. in a closed weighing bottle. Next, it was folded three times to form a rectangular parallelepiped ($1 \times 1 \times 3$ cm.) open at two ends. In this shape the membrane just fitted into the pycnometer, and air bubbles were least likely to be entrapped by the mercury. After the membrane was put in the pycnometer, any bubbles that formed were removed by inserting a thin glass rod into the mercury and allowing the bubbles to creep out along the rod. When no bubbles appeared to be present, the pycnometer was placed in the water bath and allowed to equilibrate as before. It was then taken out, wiped dry, and weighed again to 1 mg. Due to uncertainties in removing every small bubble, the variation in a series of determinations with the same membrane was as much as 10 mg. From the weight of the pycnometer without and with the membrane and the weight of the latter, the volume of the membrane was calculated. The total error in any one measurement did not exceed ± 0.5 per cent of the volume being measured.

After the weight and volume of the dried membrane were determined, the membrane was placed in water for a definite time. After this it was removed and quickly blotted dry of surface water with filter paper according to Hitchcock¹⁵ and Weech and Michaelis.⁷ It was immediately placed in the pycnometer and its volume again determined. It was then taken out, put in a closed weighing bottle, and weighed to within 0.3 mg. In this way the changes in weight and volume with time were followed until no further change occurred.

III

It immediately became apparent that the behavior of the collodion membrane in water is less simple than was generally supposed. The membranes in equi-

¹⁵ Hitchcock, D. I., *J. Gen. Physiol.*, 1925, 9, 755.

librium with water showed volume increases of 5 to 11 per cent, depending on the brand of collodion used.

Since at first sight it is not quite easy to see the true relationship of weight increases and volume increases, a sample calculation is given below.

(a) Weight increase		
Weight of membrane wet.....	0.2185 gm.	
“ “ “ dry.....	0.2040 “	
“ increase of membrane on wetting.....	0.0145 “	= 14.5 mg.
(b) Volume increase		
	Dry	Wet
Weight of membrane.....	0.2040 gm.	0.2185 gm.
“ “ pycnometer + Hg.....	189.852 “	189.852 “
“ “ “ + Hg + membrane..	188.325 “	188.216 “
Difference in weight.....	1.527 “	1.636 “
	1.527 “	1.636 “
	0.204 “	0.219 “
Weight of Hg displaced.....	1.731 “	1.855 “
Volume “ “ “	$\frac{1.731}{13.53} = 0.1280$ cc.	$\frac{1.855}{13.53} = 0.1371$ cc.
“ “ membrane.....	0.1280 cc.	0.1371 cc.
Volume of membrane wet.....		0.1371 cc.
“ “ “ dry.....		0.1280 “
“ increase of membrane on wetting....		0.0091 “ = 9.1 c. mm.

In order to visualize the quantitative meaning of these figures they must be reduced to a standard, either 1 gm. or 1 cc. of dry membrane. It is better to choose the cubic centimeter of dry material as a unit, for in this way the specific gravity factor is eliminated. Therefore the data have been recalculated for a quantity of collodion having a volume of 1 cc. in the dry state, and the weight and volume increases are expressed as milligrams and cubic millimeters respectively per 1 cc. of dry collodion. A sample calculation with the figures given before, follows:

$$\text{Weight increase of 1 cc. dry membrane on wetting (in mg.)} = \frac{0.2185 - 0.2040}{0.1280} 1000 = 113 \text{ mg.}$$

$$\text{Volume increase of 1 cc. dry membrane on wetting (in c. mm.)} = \frac{0.1371 - 0.1280}{0.1280} 1000 = 71 \text{ c. mm.}$$

In Table I are given the weight and volume measurements obtained with three membranes prepared from different brands of collodion after they had been immersed in water for 12 hours. In Fig. 1 are plotted the weight and volume increases against the time of immersion; from these curves it is seen that the values of Table I represent the equilibrium state.

TABLE I
Weight and Volume Increase of Dried Collodion Membranes on Wetting with Water

Brand of collodion	Dry weight	Wet weight	Dry volume	Wet volume	Weight increase per cc. dry membrane	Volume increase per cc. dry membrane
	mg.	mg.	c. mm.	c. mm.	mg.	c. mm.
Mallinckrodt "Parlodion"	232.5	245.0	149.2	156.7	84	50
Baker collodion U.S.P.	204.0	218.5	128.0	137.1	113	71
Schering-Kahlbaum "Celloidin"	212.2	233.0	133.3	148.0	156	110

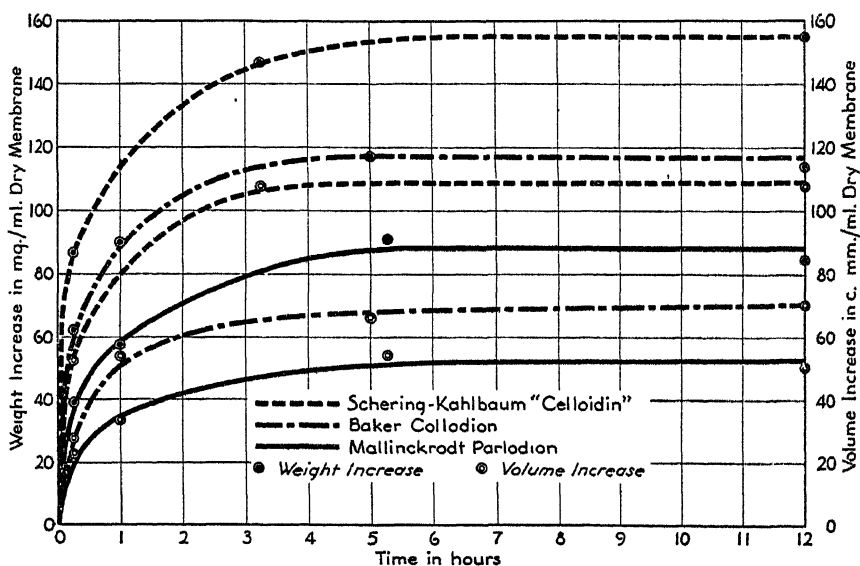


FIG. 1

Several additional experiments with Baker U.S.P. collodion indicate that membranes prepared under different conditions of drying may vary by as much as 20 c. mm. (mg.) per gm. dry collodion in their weight and volume increases. The difference of weight and volume increase, however, remains fairly constant. The differences between "Parlodion" and Baker U.S.P. collodion are still prob-

ably significant because the experiments were done at the same time under identical conditions.

It should also be mentioned here that the swelling curve for oxidized collodion was found to be identical with that of the original unoxidized preparation. Evidently the carboxyl groups that were introduced had no measurable effect on the swelling. This is in agreement with our former results³ in which it was shown that the number of carboxyl groups introduced by oxidation was relatively small.

The weight and volume changes of dried collodion which occur in wetting seem to be completely reversible. Wetted membranes were allowed to dry and were then placed in water again for a few hours. This was repeated several times. On the fifth immersion the water uptake and the swelling were exactly the same as on the first within the experimental error.

The swelling of cellulose is known to decrease with increasing temperatures; for the case of cellophane membranes this point was recently stressed by McBain and Stuewer.¹³ Since some knowledge of the temperature coefficient might possibly cast light on the mechanism of the swelling of collodion, this effect has been studied. The volumes and weights of six membranes were measured in the dry state. Then two of the membranes were put into water at 3°C., two in water at 25°C., and two in water at 50°C. After 12 hours the membranes were removed from the water and their weights and volumes again determined. From these experiments it was found that there is a very small negative temperature coefficient, the swelling at 50° being about 13 per cent less than it was at 3°.

IV

Before discussing the meaning and significance of the above results, it is best to state briefly the effects observed when a "water-wetted dried" collodion membrane is transferred from water to an electrolyte solution.

The first electrolyte solution tested was 1 M KCl, and the experimental procedure was the same as in the previous experiments. A water-wetted membrane was blotted dry, and then its volume was determined with the pycnometer. The membrane was placed in 1 M KCl for 24 hours and then removed and its volume again measured. Within the limit of experimental error there was no volume change.

Since the measurements with the pycnometer were laborious and time consuming, another method was worked out for the testing of the several electrolyte solutions to be studied. This involved the weighing of the membranes in the water-wetted state and after immersion in the solutions. Any increase or decrease in weight could be taken to indicate swelling or shrinking, provided that proper corrections are made for the specific gravity of the solutions which are taken up by the membranes.

There is considerable uncertainty as to the concentration of the solution which goes into the membrane. There is no doubt that not all of the water in a membrane is available even for KCl,¹⁶ and polyvalent ions certainly do not penetrate dried collodion membranes with ease. In view of this fact that the problem of the mean electrolyte concentration of the water in a dried collodion membrane is still unsettled, the experimental data given below are evaluated on the basis of these two assumptions:

(a) The liquid taken up by the membrane is free from electrolyte; it is salt-free water, and (b) the liquid taken up by the membrane has the same composition and therefore the same specific gravity as the particular electrolyte solution in which the membranes were immersed.

TABLE II

Weight Changes of Dried Collodion Membranes on Transference from Water to Solutions of Strong Electrolytes

1	2	3	4	5	6
Solution	Weight of membrane dry	Weight of membrane wetted with water	Weight of membrane wetted with solution	Change in weight (column 4 to column 3)	Change in weight (corrected for specific gravity)
	gm.	gm.	gm.	gm.	gm.
1 M KCl	0.528	0.563	0.563	0.000	-0.001
0.8 M K ₂ SO ₄	0.382	0.407	0.407	0.000	-0.001
1 M K ₃ Cit	0.397	0.421	0.421	0.000	-0.002
0.5 M K ₄ Fe(CN) ₆	0.355	0.380	0.378	-0.002	-0.003
1 M CaCl ₂	0.525	0.554	0.553	-0.001	-0.002
1 M AlCl ₃	0.422	0.446	0.447	+0.001	0.000
1 M KNO ₃	0.457	0.485	0.484	-0.001	-0.002
1 M KCNS	0.479	0.507	0.509	+0.002	+0.001
1 M ZnCl ₂	0.443	0.471	0.469	-0.002	-0.003
1 M Ca(CNS) ₂	0.427	0.452	0.451	-0.001	-0.002
1 M HCl	0.428	0.456	0.454	-0.002	-0.002
1 M HNO ₃	0.465	0.496	0.496	0.000	0.000
H ₂ O	0.348	0.372	0.371	-0.001	-0.001

Some situation intermediate between these two extremes is most probably the real one.

The procedure adopted for the weight method follows. The dry weights of the membranes were determined after they had been kept in a desiccator for at least 24 hours. Then they were placed in water for 24 hours; in this time equilibrium was reached. Next, they were blotted dry of surface water and transferred into a weighing

¹⁶ Unpublished experiments of the authors show that the mean concentration of KCl inside a dried membrane is always much less than in the outside solution.

bottle and their weights determined. After this the membranes were put in the electrolyte solutions being tested and allowed to remain in them for 1 week. Then they were blotted dry, and their weights were determined as before.

The specific gravity of each electrolyte solution was determined in order to apply the proper correction as discussed above.

Table II gives a summary of the results obtained with a number of representative strong electrolytes. Column 1 gives the electrolytes and their approximate concentrations; column 2 the weight of the dry membranes, column 3 the weight in the water-wet state, and column 4 the weight of the membranes after reaching equilibrium with the electrolyte solutions. Column 5 is the change in weight on transference of the membranes to the electrolyte solutions under the assumption that the liquid taken up from the electrolytes is salt-free water. Column 6 is the change in weight if the water imbibed is assumed to have the specific gravity of the outside solution.

Since the changes that occurred are within the limits of the experimental error, we may conclude that "water-wetted dried" collodion membranes do not shrink or swell significantly when transferred from water to solutions of strong inorganic electrolytes.

V

There are several obvious conclusions which can be drawn from the data that have been obtained.

The dried collodion membrane, contrary to the general assumption, swells on wetting with water. The swelling varies with the different brands of collodion, being from 50 to 110 c. mm. per cc. of dry membrane at room temperature. Oxidized collodion swells exactly to the same extent as the unoxidized preparation.

Dehydrated dried collodion membranes on wetting with water undergo fundamental changes, and therefore the air-dry and "water-wetted dried" membranes are structurally different entities. It is not permissible, as it has been done occasionally in the past, to use experimental results (gas permeability) obtained with dry membranes to explain the structure of water-wetted membranes.

The "water-wetted dried" collodion membrane does not undergo significant structural changes when transferred from water to solutions of strong inorganic electrolytes. It can be considered a rigid structure which does not swell in aqueous solutions of the typical strong inorganic electrolytes.

Although "dried" collodion membranes do swell in water, our experiments furnish additional evidence that they contain preformed pores which can take up water. In all cases studied the water uptake was in excess of the volume increase amounting to 34 to 46 mg. (or c. mm.) per cc. dry collodion. It is most likely that at least a large fraction of this difference indicates preformed

pore space in the membrane. To analyze these data further, however, one must touch at least briefly the complicated problem of the swelling of micellar substances and also the problem of "bound" water.

According to Katz¹⁷ intermicellar swelling occurs if the crystalline micelles of a colloidal material are pushed apart from each other by the swelling agent without a change of crystalline structure. Intramicellar swelling takes place if the swelling agent goes into the micelles loosening the contact of the macromolecules in the micelles and causing a change of the crystalline structure of the latter. "Bound" water in a colloidal system is defined as the water which is combined so strongly with the material that it is unavailable as solvent for other substances. As there is no sharp physical difference between "bound" and free water, different methods to determine "bound" water yield different results.¹⁸ The formation of definite hydrates consists of a very strong binding of water, and this is supplemented by additional hydration with a gradual transition towards free water. Collodion is hygroscopic, taking up considerable amounts of water from the atmosphere, even if the latter is not saturated with water vapor. This water is bound very firmly to the collodion and is not available as solvent for other substances.

If water is taken up as "bound" water, it is compressed in a manner similar to the well known volume decrease observed on mixing water and alcohol. Therefore, the difference between water uptake (in milligrams) and the volume increase (in cubic millimeters) mentioned above is not necessarily a measure of the preformed pore space; it is entirely possible that compression may account for a substantial part of this difference. This side of the problem was not investigated further, however, because it becomes more of a problem in nitrocellulose chemistry than in membrane behavior.

On the basis of the foregoing discussion and the experimental results, the following picture is tentatively suggested for the water-wetted dried collodion membrane. Intermicellar and intramicellar swelling has taken place. The individual micelles have increased in volume over the dry state; there is less cohesion between the micelles; and the micelles which were pressed together by capillary forces on drying have assumed less forced positions. These processes in all probability tend to produce intermicellar pore space and thus to increase the porosity of the membrane over whatever porosity may have existed in the air-dry state.

¹⁷ Katz, T. R., *Micellartheorie und Quellung der Zellulose*, in Hess, K., *Die Chemie der Zellulose und ihrer Begleiter*, Leipzig, Akademische Verlagsgesellschaft, 1928.

¹⁸ For a review of the problem of "bound" water the reader may consult: Gortner, R. A., *Outlines of biochemistry*, New York, John Wiley and Sons, 2nd edition, 1938, 257; other investigators (see, e.g., Blanchard, K. C., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, Long Island Biological Association, 1940, 8, 1.) are still more sceptical about the idea of bound water than is indicated in the text.

From the picture presented in the last paragraph one very definite conclusion can be reached concerning the structure of the dried collodion membrane in relation to electrolyte permeability. The problem of the water uptake of these membranes must be restated. It is not important how much water is contained in the membrane, and how much of this water is "bound" and how much of it is "free" water. In addition it is not of fundamental interest what changes the collodion membrane undergoes on wetting. The important question now becomes, how much water in the water-wetted membranes is available for the typical membrane functions. Thus the abstract question of the water content of dried collodion membranes has changed to the more concrete and better defined problem of the availability of water in the membrane in useful pathways for different solutes.

An investigation of this problem has not been carried out as yet, for the conventional dried collodion membrane is not the best membrane for such studies. More useful information may be obtained by studying specially prepared membranes of very high ionic selectivity and of a permeability several orders of magnitude larger than that of the ordinary dried membranes. Such membranes have been prepared and will be described in a later paper.

VI

Collodion membranes of high porosity, typical "porous" membranes, have also been investigated. Since the "porous" membranes do not exist in the dry state, there is only one possible change that can be measured; *i.e.*, the volume change on transfer from water to electrolyte solution. As in the case of the "water-wetted dried" membranes, it was sufficient to weigh the "porous" membranes water-wetted and after immersion in the various solutions. Changes in weight again indicate swelling or shrinkage, provided proper corrections are made for the specific gravity of the solution in the membrane.

The "porous" membranes were prepared in the same way as the "dried" ones with the exception of the drying time. In this case as soon as a tough skin had formed on the collodion (about $1\frac{1}{2}$ hours¹⁹), the surface was covered with water. In this way membranes were obtained that contained 50 to 60 per cent by volume of water and were approximately 0.2 mm. in thickness. Pieces of membrane 4×10 cm. were cut out, blotted dry, and weighed. They were then placed in the various solutions for 24 hours and, after being blotted, were weighed again. After this, the membranes were washed free of electrolyte, dried completely, and weighed once more. From this determination of the dry weight the water content was calculated, which was necessary in order to apply the correction for the specific gravity of the solution entering the mem-

¹⁹ This time was considerably longer than for the "porous" membranes described in our previous papers because the films referred to above were much thicker than those used previously.

brane. The accuracy of the method was only ± 0.6 per cent, but this was sufficient for our purpose. Changes in porosity within these limits could hardly influence the behavior of "porous" membranes to a significant extent.

In Table III results are given for the same series of electrolytes that were used in the study on the dried membranes. In this case only the values that have been corrected for the specific gravity are reported since in the porous membranes, the electrolyte concentration in the membrane approaches that of the outside solution.

TABLE III

Weight Changes of Porous Collodion Membranes on Transference from Water to Solutions of Strong Electrolytes

1	2	3	4
Solution	Weight of membrane wetted with water	Weight of membrane wetted with solution (corrected for specific gravity)	Change in weight (column 3 to column 2)
	gm.	gm.	gm.
1 M KCl	0.906	0.902	-0.004
0.8 M K ₂ SO ₄	0.687	0.685	-0.002
1 M K ₃ Cit	0.851	0.845	-0.006
0.5 M K ₄ Fe(CN) ₆	0.921	0.908	-0.013
1 M CaCl ₂	0.691	0.689	-0.002
1 M AlCl ₃	0.659	0.654	-0.005
1 M KNO ₃	0.678	0.677	-0.001
1 M KCNS	0.646	0.649	+0.003
1 M ZnCl ₂	0.583	0.582	-0.001
1 M Ca(CNS) ₂	0.778	0.776	-0.002
1 M HCl	0.734	0.730	-0.004
1 M HNO ₃	0.865	0.865	0.000
H ₂ O	0.761	0.760	-0.001

As with the dried membranes there is no detectable change in volume on the transference of "porous" membranes from water to strong electrolytes. Within these limits, at least, the structure of these "porous" membranes can be considered to be rigid and permanent.

As in the case of the dried membranes the problem next to be studied is not the total water content of these membranes, but how much of this water is available for electrolytes, and how large a fraction of this exists in useful pathways. It will not be as complex as in the former instance, because undoubtedly most of the water in the "porous" membranes exists in large pores accessible to almost all electrolytes. This problem can only be solved, however, by further studies on the permeability, electrolyte content, and resistance of collodion membranes of varying porosity.

SUMMARY

1. The assumption has generally been made that collodion membranes are rigid and non-swelling in water and aqueous solutions of strong electrolytes, and considerable uncertainty exists as to the manner in which water is taken up by "dried" collodion membranes. In approaching these problems experimentally, the weight and volume changes of collodion membranes when placed in water and when transferred from water to solutions of strong electrolytes were determined.

2. Dried collodion membranes swell reversibly to an appreciable extent when placed in water, the swelling varying from 5 to 11 per cent depending on the brand of collodion. The water uptake and swelling of oxidized collodion is the same as the original unoxidized preparation.

3. The water uptake as determined by the weight increase is larger than could be accounted for by the volume increase, swelling accounting for only 60 to 70 per cent of the water taken up by the membranes.

4. When the "water-wetted dried" collodion membranes were transferred from water to solutions of various strong electrolytes, there was no detectable change in volume. Similarly, when the "porous" membranes were transferred from water to solutions of strong electrolytes, there was no significant volume change.

5. When dried collodion membranes swell in water, some of the water becomes "bound" water, and both intramicellar and intermicellar swelling seem to occur. Therefore, neither the weight increase nor the volume increase nor their difference can be taken as a measure of the true pore space of the membrane. It is concluded that the important problem is not the absolute water content, but how much water in the water-wet membranes is available in useful pathways for the different solutes.

A MODEL OF THE POTASSIUM EFFECT

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The protoplasm of certain cells has to a marked degree the power to distinguish electrically between Na^+ and K^+ . This ability has been called the "potassium effect."

It is clearly seen when we replace¹ 0.01 M KCl in contact with a *Nitella* cell by 0.01 M NaCl. We then observe a change of P.D. in a positive² direction by an amount which varies from 30 to 95 mv.³

We find a pronounced potassium effect in *Valonia*⁴ and in *Halicystis*.⁵ Such an effect has long been known in muscle⁶ and is also found in nerve.⁷

The potassium effect can be removed from *Nitella* by leaching in distilled water and thus removing a substance which for convenience has been called *R*. This substance can be recovered from the water and applied to the cell and this restores⁸ its ability to distinguish electrically between K^+ and Na^+ . To do this we remove the water from the cells and shake it with petroleum ether which is then drawn off and evaporated to dryness: the residue is then taken up in a relatively small quantity of distilled water which is applied to the cell. The potassium effect is promptly restored.

It would therefore appear that the *Nitella* cell contains a substance which is soluble in petroleum ether and which is responsible for the potassium effect.

A variety of organic substances has been investigated with a view to making a model of the potassium effect. The most interesting thus far encountered is nitrobenzene. When this is shaken with M/1 KCl and allowed to stand in

¹ Replacing 0.01 M NaCl by 0.01 M KCl may produce an action current and thus make the interpretation more difficult.

² I.e., the spot in contact with 0.01 M NaCl becomes positive (in the external circuit) to a spot in contact with 0.01 M KCl.

³ Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 715. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312. Also many unpublished measurements.

⁴ Damon, E. B., *J. Gen. Physiol.*, 1932-33, **16**, 375.

⁵ Blinks, L. R., *J. Gen. Physiol.*, 1932-33, **16**, 147. Osterhout, W. J. V., *J. Gen. Physiol.*, 1937-38, **21**, 631.

⁶ Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, Wilhelm Engelmann, 6th edition, 1926, 649.

⁷ Cowan, S. L., *Proc. Roy. Soc. London, Series B*, 1934, **116**, 216.

⁸ Osterhout, W. J. V., and Hill, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1934-35, **32**, 715. Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, **23**, 429.

contact with it we find that replacement of $m/1$ KCl at any spot by $m/1$ NaCl causes a change of potential in a positive direction (as in *Nitella*)⁹ amounting to about 67 mv. This compares favorably with the values found for *Nitella* and is higher than that of 49 mv. reported by Beutner¹⁰ for *m*-nitrobenzene dissolved in nitrobenzene.

When nitrobenzene, previously shaken with $m/1$ aqueous KCl, is placed in contact with aqueous $m/1$ KCl on one side and with aqueous $m/1$ NaCl on the other we may assume that the situation resembles that shown in Scheme 1. Here B'' and C' are very thin layers on each side of the phase boundary and the customary assumption is made that they at once come into approximate equilibrium with each other. This involves a movement of KCl from B'' to C' and a movement of NaCl from C' to B'' .

Aqueous		Non-aqueous			Aqueous	
A	A'	B'	B	B''	C'	C
KCl	KCl	KCl	KCl	KCl	NaCl	NaCl
$m/1$	$m/1$	m/X	m/X	$< m/X$	+	$m/1$
				+ NaCl	KCl	
		P ₁		P ₂		P ₄

SCHEME 1

At the other phase boundary the corresponding layers A' and B' are in equilibrium from the start: the concentration of KCl in B' is probably less than $m/10^6$ but as it is not definitely known it has been designated as m/X .

Evidently KCl will diffuse from B into C and NaCl will diffuse from C into B. Hence we may expect diffusion potentials at P_2 and P_4 .

If C' contained $m/1$ KCl and no NaCl and the adjacent region of C contained $m/1$ NaCl and no KCl the potential might be computed from the formula¹¹ (for 25°C.)

$$P_4 = 59 \log \frac{\Delta_{\text{KCl}}}{\Delta_{\text{NaCl}}}$$

in which Δ_{KCl} and Δ_{NaCl} are the equivalent conductivities of $m/1$ KCl and $m/1$ NaCl in aqueous solution. Lacking a satisfactory value for Δ_{KCl} ÷

⁹ The spot in contact with NaCl is positive in the external circuit to the spot in contact with KCl.

¹⁰ Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, Ferdinand Enke, 1920, 32.

¹¹ Lewis, G. N., and Sargent, L. W., *J. Am. Chem. Soc.*, 1909, **31**, 363. MacInnes, D. A., Principles of electrochemistry, New York, Reinhold Publishing Corporation, 1939, 233.

Δ_{NaCl} at 25°C. we may use the value at 18°C. which must be almost the same. We then have¹²

$$P_4 = 59 \log \frac{98.08}{74.19}$$

$$= 7.2 \text{ mv.}$$

Evidently the actual value is much less since C' must contain a good deal of NaCl.

We may say that $P_1 + P_2 + P_3 + P_4 = 67 \text{ mv.}$ and if P_4 is less than 7.2 mv. the value of $P_1 + P_2 + P_3$ must be more than 60 mv. since the potential at P_4 makes NaCl more positive to KCl in the external circuit and thus has the same direction¹³ as the total potential of 67 mv.

Aqueous		Non-aqueous			Aqueous	
A	A'	B'	B	B''	C'	C
KCl	KCl	KCl	KCl	KCl	KCl	KCl
m/1	m/1	m/X	m/X	<m/X	>m/10	m/10
		P_1		P_2	P_3	P_4

SCHEME 2

At P_2 we have a diffusion potential in the nitrobenzene whose value depends on the mobilities and activities of the ions involved. In order to get information regarding mobilities measurements were made of the concentration potentials.

For this purpose nitrobenzene was shaken¹⁴ with m/1 KCl and placed in contact with aqueous m/1 KCl on one side and with aqueous m/10 KCl on the other. We may assume that the situation is like that in Scheme 2. The observed p.d. is $25 \pm 0.5 \text{ mv.}$ (4 observations) with the dilute solution positive in the external circuit.

The maximum possible value of P_4 would occur if C' contained m/1 KCl and the adjacent region of C contained m/10 KCl. We should then have¹⁵ at 25°C.

$$P_4 = 59 (2t_K - 1) \log \frac{a_1}{a_2}$$

¹² The values for conductivity at 18°C. are taken from International Critical Tables, 1929, 6, 233, 234.

¹³ This might apply to a certain extent to a *Nitella* cell treated first with 0.01 M KCl and then with 0.01 M NaCl.

¹⁴ In order to increase the conductivity of the nitrobenzene as much as possible it was shaken with the more concentrated solution.

¹⁵ MacInnes,¹¹ p. 225.

where t_K is the transference number of K^+ and a_1 and a_2 are the mean ionic activities of the two solutions of KCl. The average transference number¹⁶ of KCl at 25°C. between $m/1$ and $m/10$ is 0.4885. Inserting this value and putting for convenience activities equal to concentrations we have

$$P_4 = 59(2[0.4885] - 1) \log \frac{1.0}{0.1} \\ = -1.4 \text{ mv.}$$

This would tend to make the total potential less and hence we might correct for it by adding 1.4 mv. but the value is undoubtedly less than 1.4 mv. and it may well be neglected.

In experiments with guaiacol it has been found¹⁷ that the value of the phase boundary potentials $P_1 + P_3$ could be neglected. If, for purposes of calculation, we assume that this is the case here we may say that P_2 equals 25 mv. We then have¹⁷ for 25°C.

$$P_2 = 25 = 59 \frac{u_K - v_{Cl}}{u_K + v_{Cl}} \log \frac{a_1}{a_2}$$

where u_K is the mobility of K^+ and v_{Cl} that of Cl^- in nitrobenzene, and a_1 and a_2 are the mean ionic activities corresponding to the two concentrations of KCl in nitrobenzene. In view of results previously obtained with guaiacol¹⁷ we may assume that $a_1 \div a_2 = 1.0 \div 0.1$; i.e., is equal to the ratio of the aqueous concentrations.

For convenience we may put the mobility of Cl^- equal to unity and designate it as \bar{v}_{Cl} and the corresponding value of K^+ as \bar{u}_K . We then have

$$P_2 = 25 = 59 \frac{\bar{u}_K - 1}{\bar{u}_K + 1} \log \frac{1.0}{0.1}$$

whence $\bar{u}_K = 2.5$. This means that in nitrobenzene $u_K \div v_{Cl} = 2.5$.

Proceeding in the same way with NaCl we shake nitrobenzene with $m/1$ NaCl and place it in contact with $m/1$ NaCl on one side and with $m/10$ NaCl on the other and obtain 31 ± 1.5 mv. (4 observations) with the dilute solution positive in the external circuit. In this case we may expect a larger value at P_4 . The maximum possible value would occur if C' contained $m/1$ NaCl and the adjacent region of C contained $m/10$ NaCl. In that case we should have¹⁸ (putting for convenience activities equal to concentrations)

$$P_4 = 59(2t_{Na} - 1) \log \frac{1.0}{0.1} \\ = 59(2[0.379] - 1) \\ = -14.3$$

¹⁶ MacInnes, D. A., and Dole, M., *J. Am. Chem. Soc.*, 1931, **53**, 1357.

¹⁷ Osterhout, W. J. V., *J. Gen. Physiol.*, 1942-43, **26**, 293.

¹⁸ The value of $t_{Na} = 0.379$ is the average of t_{Na} at $m/10 = 0.385$ (MacInnes,¹¹ p. 85) and t_{Na} at $m/1 = 0.373$ (MacInnes, personal communication).

Here t_{Na} is the average transference number of Na^+ . This value of P_4 is undoubtedly much larger than the actual value since the concentration in C' would fall off rapidly because NaCl would diffuse away from C' faster than it diffused into it. The diffusion in the nitrobenzene is relatively slow both because of the very low concentration gradient¹⁹ and the higher viscosity.²⁰

This potential would be in the opposite sense to that in the nitrobenzene, *i.e.* it would tend to make the dilute solution negative since in aqueous solutions u_{Na} is less than v_{Cl} , and if a correction were made it would involve an addition to the observed value of 31 mv.²¹

These results appear to indicate that in nitrobenzene u_{Na} may be somewhat greater than u_{K} . Hence the fact that KCl is negative to NaCl in the external circuit cannot be due to the difference in ionic mobilities for this would make KCl positive. But it would seem that this result should be accepted with reserve in view of the fact that in guaiacol,¹⁷ where the situation appears to be quite clear, u_{K} is greater than u_{Na} (but the opportunity of the ion to surround itself with water is greater in guaiacol since it contains much more water).

The observed result might be explained if the ionic activity of KCl in nitrobenzene were very much greater than that of NaCl . An attempt was accordingly made to ascertain the relative concentrations but the solubility of KCl and of NaCl in nitrobenzene is too small to admit of satisfactory determination.

Since the salicylates²² are more soluble in nitrobenzene some measurements were made with them. The partition coefficient S (S = concentration of K^+ or Na^+ in nitrobenzene divided by that in the aqueous solution) is about 0.000012 for $m/1$ Na -salicylate and about 0.00014 for $m/1$ K -salicylate; *i.e.*, the latter is about 11.7 times as great.²³ This would not account²⁴ for the po-

¹⁹ In C' and C the maximum possible concentration gradient would occur if C' contained a little less than $m/1$ NaCl : in this case the concentration gradient in nitrobenzene would be nearly zero.

²⁰ The viscosity of nitrobenzene is more than twice that of water. According to Landolt-Börnstein's *Physikalisch-chemische Tabellen*, 1912, 1, 132, η for nitrobenzene at 25°C . is 0.01834 and for water 0.00895 (p. 135). Cf. *International Critical Tables*, 1930, 7, 217 and 1929, 5, 10.

²¹ In the case of a *Nitella* cell treated first with 0.01 m NaCl and then with 0.001 m NaCl somewhat similar considerations may apply.

The value obtained in this case is usually about 25 mv. (Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, 23, 429) which does not differ much from that found with nitrobenzene. The value with *Nitella* for 0.01 m vs. 0.001 m KCl ranges from 26 mv. (Osterhout, W. J. V., *J. Gen. Physiol.*, 1939-40, 23, 429) to about 50 mv. (Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715).

²² K -salicylate and Na -salicylate act much like KCl and NaCl with *Nitella*.

²³ These values are for K^+ and Na^+ : the values for total salicylate are higher since salicylic acid is taken up in addition to K -salicylate and Na -salicylate.

²⁴ This is evident when we employ Henderson's equation. Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1938-39, 22, 139.

tential of M/1 K-salicylate *vs.* M/1 Na-salicylate with nitrobenzene,²⁵ which is 56 ± 3 mv. (6 observations) even if we regard u_{Na} and u_K as equal.²⁶

It would take a much greater difference in partition coefficients to account for the 67 mv. found for M/1 KCl *vs.* M/1 NaCl. The actual value of the partition coefficient is not known but it was found that the conductivity of nitrobenzene when shaken with M/1 KCl is much higher than when shaken with M/1 NaCl. The interpretation is complicated by the possibility that the amount of water taken up may not be equal in both cases.²⁷

It may be asked whether KCl in nitrobenzene produces proportionally more ions²⁸ than NaCl, possibly by the formation of complex ions of the type $(KX)_I^+$, $(KX)_II^+$, etc. Such ions have been postulated in certain cases by Fuoss²⁹ and by Kraus.³⁰ To account for the observed value of 67 mv. by the formation of simple ions the dissociation constant of KCl in nitrobenzene would have to be much greater than that of NaCl.

To what extent phase boundary potentials may enter into the observed values cannot be determined at present.

An interesting parallel between the model and *Nitella* is seen in the effect of guaiacol. In the model when nitrobenzene is replaced by guaiacol (arranged as in Scheme 1, page 92) the potassium effect is only 16 mv. It is therefore not surprising that an admixture of guaiacol with nitrobenzene in the model causes the potassium effect to fall off markedly. The application of guaiacol to *Nitella* and to *Valonia* also causes a falling off in the potassium effect.³¹

There is still another resemblance between *Nitella* and the model. If in Scheme 1 we substitute various chlorides in the model we find that the mobilities³² fall off in the following order



²⁵ As with the chloride, K^+ is negative in the external circuit to Na^+ .

²⁶ The concentration effect of K-salicylate and Na-salicylate with nitrobenzene (M/1 *vs.* M/10 in each case) is about 13 mv. (set up as in Scheme 2). The interpretation is rendered doubtful because we do not know the value of P_4 and because the total salicylate taken up by the nitrobenzene is more than twice as great as the amount of K-salicylate or Na-salicylate.

²⁷ The taking up of water increases the conductivity though to a much less extent than in guaiacol (regarding guaiacol see Shedlovsky, T., and Uhlig, H. H., *J. Gen. Physiol.*, 1933-34, **17**, 549).

²⁸ The dissociation in nitrobenzene is evidently less than in water since the dielectric constant of nitrobenzene at 20°C. is 36.1 (International Critical Tables, 1929, **6**, 92).

²⁹ Fuoss, R. M., *Chem. Rev.*, 1935, **17**, 27.

³⁰ Kraus, C. A., *Tr. Electrochem. Soc.*, 1934, **66**, 179.

³¹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, **22**, 417; 1939-40, **23**, 171; 1936-37, **20**, 13.

³² CsCl is more negative to KCl than it is to RbCl and KCl is less negative to NaCl than it is to LiCl. The values cannot be accounted for by the potentials in the aqueous solutions.

This is the order of mobilities in water.³³ The same order is found in *Nitella*³¹ except that Rb and K are equal and Cs comes at the bottom of the list instead of at the top: a somewhat similar behavior of Cs is not unusual in living cells.³¹ NH₄ comes between K and Na, as in *Nitella*. (In water it comes between Rb and K.)

In comparing the model with *Nitella* we must bear in mind that the changes of potential due to the application of salt solutions are very rapid³⁴ and therefore affect only the outer non-aqueous surface layer of the protoplasm. We suppose that when this layer is allowed to stand for a short time in contact with KCl it comes into equilibrium with it since it is less than a micron in thickness. Hence the effect is much the same as when the layer of nitrobenzene is shaken with KCl. When KCl in contact with *Nitella* is replaced by NaCl we suppose that the changes which occur in this layer are similar to those discussed in connection with Scheme 1 (page 92).

Methods

Nitrobenzene (Eastman Kodak Company No. 387) was shaken with 5 per cent NaOH solution and washed with distilled water. Then followed washings with 5 per cent H₂SO₄ and finally several washings with distilled water. The sample was then carefully distilled and the fraction coming over at 208.5°C. (757 mm. of Hg) was collected. It had a very pale amber color.

In preparing the equilibrium mixtures for the concentration effect of KCl about 500 cc. of molar KCl solution were shaken with 25 cc. of nitrobenzene on a shaking machine for 3 hours. A like volume of M/10 KCl solution was shaken with a few cubic centimeters of nitrobenzene at the same time to insure saturation with nitrobenzene.

The solutions were shaken at a temperature of $25 \pm 1^\circ\text{C}$. and kept at this temperature overnight. The nitrobenzene was then drawn off, filtered through glass wool and then through a high grade of filter paper to remove droplets of the aqueous solution. It was then ready for use.

A similar procedure was used in studying the concentration effect of NaCl. In studying the chemical effect (molar KCl vs. molar NaCl) the nitrobenzene was shaken with the molar KCl solution.

A similar procedure was followed with the salicylates. The salicylate stock solutions were prepared only as needed and were protected from direct light. The salicylates used were supplied by the Monsanto Chemical Company and Merck and Company. There was no great difference in the values obtained with the two samples nor with samples which were purified by recrystallization three times from ethyl alcohol.

Measurements of potential were made in a variety of ways including (a) U-tubes

³³ Taylor, H. S., Physical chemistry, New York, D. Van Nostrand Co., Inc., 2nd edition, 1930, 1, 673. The position of Cs may be just below Rb (see International Critical Tables, 1929, 6, 231).

³⁴ Hill, S. E., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1938-39, 22, 107.

with nitrobenzene at the bottom and aqueous solutions above,³⁵ and (b) curved Pyrex tubes closed at the ends by fusing on porous fritted disks of Pyrex glass; these tubes were filled with aqueous solutions and placed in beakers filled with nitrobenzene so that the porous glass disks were parallel in a vertical position and only a few millimeters apart, thus reducing the resistance.

The most reproducible results were obtained by using glass tubes with open ends dipping in nitrobenzene as described in a previous paper.¹⁷ In each tube was a stopcock³⁶ which was kept closed during the measurements in order to keep the aqueous solution in place. These tubes were connected by means of calomel electrodes to a Compton electrometer. All the results reported here were obtained in this way.

The aqueous solutions were saturated with nitrobenzene in all cases (and contained guaiacol when this was added to the nitrobenzene).

The calomel electrodes were made up with 3.5 M KCl and a very pure electrolytic calomel. They were tested for asymmetry potential before each experiment. If the asymmetry potential was more than 0.5 mv. the pair was not used. In most cases no asymmetry potential was detectable.

All measurements were made with the Compton electrometer (Cambridge Instrument Co.) used as a null instrument and a potentiometer (Leeds and Northrup student type). The standard cell used for the reference E.M.F. and calibration was a Weston Model 4 standard cell. This cell was checked at 25°C. against a precision laboratory standard cell. The voltage was 1.01833 volts. The working battery consisted of storage cells in series. The potentiometer was checked against the standard cell at the start and at the finish of each set of experiments.

The electrometer switch was a rebuilt Leeds and Northrup single pole-double throw tapping switch. The metal contact arms were removed and mounted on a thick piece of clear Bakelite and mounted by stand-off insulators on another piece of Bakelite. The switch handle was replaced by a 6-inch length of lucite rod. In this manner surface leakage was reduced to a minimum and erratic movements of the electrometer were eliminated. The switch, potentiometer, and rheostat were all mounted in a shielded box and worked from without by means of extension controls. The cables to the cells and electrometer were Belden crystal microphone shielded cable, the shield being grounded at several points wherever it was more than 4 feet in length. All cables were grounded at both ends. The insulation resistance of this wire was checked on the General Radio Type 544-B megohm bridge and gave a resistance of "infinity" measured from the inner conductor to a point on the outer insulation.

The cells were set up in a grounded metal cabinet maintained by a thermometer relay at $25 \pm 0.1^\circ\text{C}$., although experiments in which the temperature was allowed to change as much as 5°C . showed no marked degree of change in E.M.F. due to thermal change. The conductivity measurements were made at the same temperature.

If a cell behaved erratically, its resistance was checked on the General Radio megohm bridge. In practically all cases of erratic behavior, the cell showed a resistance of the order of 300 or 400 megohms or higher. The cause was usually poor

³⁵ Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1932-33, **16**, 157.

³⁶ No stopcock grease was employed. It may be noted that stopcocks in nitrobenzene should be avoided.

contact around the stopcock junction. (A check of the potential across the stopcocks using a symmetrical chloride cell, $M/1$ NaCl vs. $M/1$ NaCl, showed the potential to be negligible.)

The average resistance for the chloride cells was about 20 megohms; for the salicylates about 800,000 ohms.

If the measurement of potential changed more than 5 mv. in the course of an hour the measurement was rejected.

In determining the distribution of sodium and potassium salicylates between water and nitrobenzene the following reagents were employed.

Potassium salicylate (Eimer and Amend, "pure"); recrystallized from ethanol, washed with ether, and dried at room temperature under reduced pressure.

Sodium salicylate (Merck's reagent grade).

HCl 0.1 M ; prepared from redistilled C.P. acid.

The solutions were shaken for about 3 hours in a mechanical shaker, at 25°C., allowed to stand overnight, separated, and the nitrobenzene layer filtered through washed and dried Pyrex glass wool.

Determination of Na and K.—Since the chlorides are much less soluble than the salicylates in nitrobenzene the cation in the nitrobenzene phase was extracted by shaking with 0.1 M HCl. The aqueous extract was evaporated to dryness in a platinum dish, dried overnight at 110°C. to drive off salicylic acid and excess HCl. The dried residue was dissolved in a small volume of water and any excess acid was determined by titration with dilute alkali; then the chloride was determined by potentiometric titration, using $AgNO_3$. The cation concentration was assumed to be equal to the corrected chloride concentration. A check determination was made by evaporating the aqueous extract, drying at 110°C., igniting at about 400°C., and weighing the residue on a micro balance.

The cation in the aqueous phase was determined by conversion of the salicylate to the chloride and weighing.

Salicylate was determined by means of the colored solution produced by the reaction between ferric ion and salicylic acid. To 10.0 ml. of the neutral salicylic solution was added 0.4 ml. of a solution containing 0.2 N $Fe_2(SO_4)_3$ + 0.1 N H_2SO_4 and the concentration determined by means of a photoelectric photometer with a filter having a maximum transmission at 5600 Å. The color intensity did not vary appreciably between 5 and 60 minutes from the time of mixing.

The salicylate in the nitrobenzene was determined after extraction with dilute NaOH (0.001 to 0.002 N).

The conductivity of the nitrobenzene was measured in a Wheatstone bridge with a conductivity cell, having electrodes of platinized platinum. Alternating current at 1000 cycles was employed.

All measurements were made at $25 \pm 0.1^\circ C$.

(Since the vapor of nitrobenzene is toxic good ventilation was provided so that the odor of nitrobenzene did not become noticeable.)

I wish to express my thanks to Mr. Donald J. DeCain and Mr. Harry Bodner for the care and skill with which they have made the measurements.

SUMMARY

The protoplasm of certain cells is able to distinguish electrically between K^+ and Na^+ . This has been called the potassium effect.

This is illustrated by experiments with *Nitella*. When 0.01 M KCl which has stood in contact with *Nitella* is replaced by 0.01 M NaCl the p.d. changes in a positive direction by an amount which varies between 30 and 95 mv. This ability to distinguish between K^+ and Na^+ disappears with the removal of an organic substance from the cell. The amount of this substance is doubtless too small to make it possible to obtain enough for analysis. An attempt has therefore been made to find an organic compound which can produce similar effects.

It is found that when M/1 KCl in contact with nitrobenzene (previously shaken with M/1 KCl) is replaced by M/1 NaCl the potential changes in a positive direction to the extent of 67 mv. which compares favorably with the values found in *Nitella*.

This is not due to a greater mobility in nitrobenzene of K^+ as compared with Na^+ : this is evident from measurements of concentration effects with nitrobenzene (M/1 KCl vs. M/10 KCl and M/1 NaCl vs. M/10 NaCl). It might be brought about if KCl produced in nitrobenzene a sufficient preponderance of ions (simple or complex) as compared with NaCl. Whether this occurs could not be determined but it was found that nitrobenzene shaken with M/1 KCl has a higher conductivity than when shaken with M/1 NaCl.

Measurements with salicylates showed that K-salicylate has a partition coefficient about 11.7 times as great as that of Na-salicylate. It was also found that when M/1 K-salicylate in contact with nitrobenzene (previously shaken with M/1 K-salicylate) is replaced by M/1 Na-salicylate there is a change of potential in a positive direction amounting to 56 mv.

To what extent phase boundary potentials may enter into the observed values cannot be determined at present.

The model resembles the *Nitella* cell in that RbCl and KCl are negative to NH_4Cl which in turn is negative to NaCl and still more so to LiCl (in the model CsCl is negative to KCl but in *Nitella* it is positive).

It likewise resembles *Nitella* in that the potassium effect is lessened by the addition of guaiacol.

DENATURATION CHANGES IN EGG ALBUMIN WITH UREA, RADIATION, AND HEAT

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The change produced in native proteins by various agents which results in loss of their characteristic properties and changes in solubility is called denaturation. The term, however, may be applied to structural and physical changes in the protein molecule which differ widely with the particular agent used.

Urea denaturation differs in many respects from both heat and radiation denaturation. Urea has a strong dispersive¹ action on proteins so that when a high concentration of urea is added to a protein the solution remains clear until the solution is diluted or dialyzed when a certain amount of insoluble protein is formed. Hopkins (1) found that the rate of denaturation of egg albumin by urea varied inversely with the temperature, but Diebold (2) found a positive temperature coefficient for the denaturation of fibrinogen by urea. Owing to its dispersive action high concentrations of urea will dissolve proteins coagulated by the action of heat or radiation, or other denaturing agents. Laporta (3) reports a certain amount of reversal of denaturation as a result of this dispersion by urea. Steinhardt (4) has shown that the functional properties of hemoglobin and pepsin are retained in urea solution so that the loss of solubility after treatment with urea may not be a true denaturation.

Many observers (2, 5, 6) have found that sulfhydryl groups appear after denaturation which are not detectable in native proteins. In the case of urea denaturation the number of SH groups is independent of the protein concentration and depends on the concentration of urea.

In the presence of urea most proteins split into smaller molecules. Burk and Greenberg (7) found that in 40 per cent urea hemoglobin had half of its normal molecular weight but that egg albumin was unchanged. Recently Williams and Watson (8) found some dissociation of egg albumin into smaller molecules in 50 per cent urea.

This investigation was undertaken with the idea of comparing the effect of different denaturation agents on molecular structure and shape and of studying the interrelations between the effects of different denaturation agents. In the course of the study certain new observations on urea denaturation were made.

¹ The term "dispersion" is used here to denote the opposite of "aggregation" as in the articles by Hopkins (1).

Methods

The material used was isoelectric egg albumin (0.9 per cent except where otherwise stated) prepared by the method given in previous publications (9, 10).

Ultraviolet radiation was given by the General Electric Portable A.C. Uviarc.

Measurements of the opalescence of the solution were made by means of the Tyndall meter and Macbeth illuminometer described in a previous publication (9) and in observations of the depolarization of the Tyndall beam a polarizing eye piece was used with the Macbeth illuminometer and readings taken with the nicol in two positions. The ratio of the dark to the bright component multiplied by 100 gave the per cent depolarization.

Optical rotation measurements were made with a Hilger polarimeter giving readings to 0.01° . The *D* line of sodium was used throughout and the experiments were carried out at room temperature.

Hydrogen ion determinations were made colorimetrically.

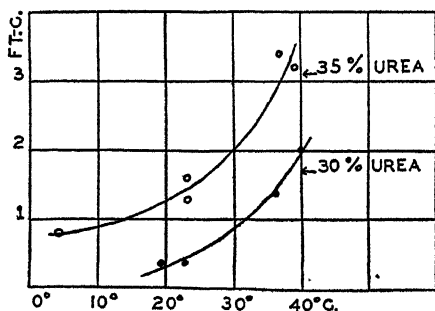


FIG. 1. Effect of temperature on rate of urea denaturation of egg albumin. Ordinates = opalescence of Tyndall beam in apparent foot-candles. Abscissae = temperature.

RESULTS

Urea Denaturation.—When urea is added to isoelectric egg albumin solutions in concentrations of 10 to 50 per cent there is a shift of pH to about pH 5.2–5.8 depending on the concentration of urea. Even if these solutions are brought to pH 4.8 they remain clear as long as the urea is present.

If the urea is dialyzed out and the solutions adjusted to the isoelectric point there is a precipitate formed and the degree of opalescence was taken as a measure of the denaturation. Contrary to the observations of Hopkins (1) the rate of denaturation was found to have a positive temperature coefficient, the rate increasing rapidly with temperature above 20°C . The results are given in Fig. 1 and Table I for solutions standing 2 hours at temperatures from 4 – 40°C .

The rate of denaturation at 40°C . for different concentrations of urea is shown in Fig. 2. The urea was added to isoelectric albumin bringing the pH to 5.4–5.6

and subsequently dialyzed and adjusted to pH 4.8. With concentrations of urea less than 20 per cent the rate of denaturation was too slow to be appreciable.

TABLE I
Temperature Coefficient of Denaturation of 0.9 Per Cent Egg Albumin by Urea

Temperature °C.	Temperature coefficient	
	30 per cent urea	35 per cent urea
10-20	—	1.33
20-30	2.0	1.66
30-40	2.5	2.0

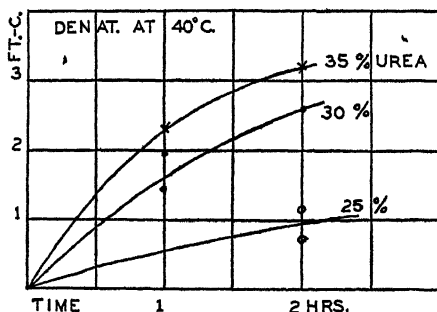


FIG. 2. Rate of urea denaturation of egg albumin at 40° C. Ordinates = opalescence of Tyndall beam in apparent foot-candles Abscissae = time of immersion in water bath at 40° C.

TABLE II

pH	Urea concentration	Opalescence	
		6 days 21°	7 days 4°
		<i>ft.-candles</i>	<i>ft.-candles</i>
4.8	25	2.35	0.41
	30	3.70	1.38
	40	5.30	2.00

Although urea denaturation is very slow at 0° after 9 to 10 days in the ice box the following results are obtained (see Table II).

One may conclude, therefore, that 20 per cent urea produces practically no denaturation in a 0.9 per cent egg albumin solution. Urea denatures slowly at a concentration of 25 per cent. 35 per cent is rapidly effective at room temperature and 30 per cent is rapidly effective at a somewhat higher temperature.

Conflicting results on urea denaturation are undoubtedly due to the fact that the concentration necessary to produce denaturation varies with the protein used and its concentration as well as with the concentration of urea and the temperature of the solution.

Effect of Urea on Heat Denaturation.—It has been stated that urea, because of its dispersive action prevents the heat coagulation of proteins. It was found that in the presence of 50 per cent urea, egg albumin solutions brought to boiling will not precipitate until the urea is dialyzed out and the pH adjusted to 4.8. Also it was found that heat-coagulated albumin will redissolve in 50 per cent urea. However, the protein is dispersed without reversal of denaturation.

Concentrations of 20 to 40 per cent urea did not prevent the heat denaturation of albumin and indeed seemed to accelerate the denaturation. If tubes con-

TABLE III
Effect of Urea on Heat Denaturation

pH	Urea <i>per cent</i>	Temperature		Opalescence
				<i>ft.-candles</i>
4.8	—	58° C.	$\frac{1}{2}$ min.	—
4.8	25	58° C.	$\frac{1}{2}$ min.	5.2
4.8	30	58° C.	$\frac{1}{2}$ min.	6.2
4.8	40	58° C.	$\frac{1}{2}$ min.	6.2
4.8	50	58° C.	$\frac{1}{2}$ min.	—
4.8	—	58–60° C.	10 min.	1.8
4.8	25	58–60° C.	10 min.	4.7
4.8	—	62° C.	3 min.	1.78
4.8	25	62° C.	3 min.	4.4

taining egg albumin only and egg albumin plus 25, 30, and 40 per cent urea, readjusted to pH 4.8, were heated simultaneously, opalescence appeared first in the tubes containing 30 and 40 per cent urea. These show some opalescence as the temperature of the water in which the tubes are immersed reaches 54° C. If the water is brought to 58° C. and the tubes then removed the tube containing egg albumin is still clear and those containing urea show a dense precipitate, the precipitate being heavier in the tubes containing 30 and 40 per cent than in the tube containing 25 per cent.

Some results are summarized in Table III.

This would seem to indicate that urea in concentrations too low to split the molecule and disperse the heat-denatured protein lowers the heat coagulation temperature of egg albumin and accelerates the rate of heat coagulation. Actually a different interpretation was found to be more consistent with the facts as it is probable that heat below the temperature of heat coagulation breaks the urea-protein complex and allows the precipitation of urea-denatured protein.

Effect of Urea on Radiation Denaturation.—In a previous publication (9) it was stated that egg albumin exposed to ultraviolet radiation at 4° C. remained clear until heated for a short time to a moderate temperature (40° C.). With the stronger arc used in these experiments, and consequently less perfect temperature control, there was slight opalescence after radiation on ice, although this opalescence was prevented by adding 25 to 30 per cent urea to the solutions. The zone in which opalescence occurs is also narrowed in the presence of urea.

If the solutions are radiated on ice and then put in a water bath at 40° C. the opalescence that normally occurs is diminished by 25 per cent urea and completely prevented by 35 per cent (see Fig. 3).

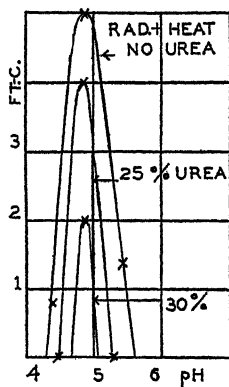


FIG. 3

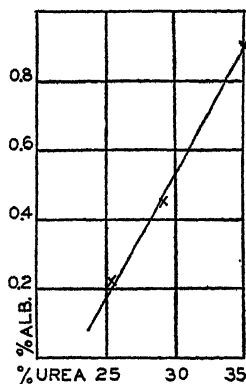


FIG. 4

FIG. 3. Effect of urea on denaturation by radiation followed by heating to 40° C. Ordinates = opalescence of Tyndall beam in apparent foot-candles. Abscissae = pH.

FIG. 4. Concentration of urea necessary to prevent aggregation in egg albumin after radiation and heat (40° C.) at the isoelectric point. Ordinates = concentration of albumin in per cent. Abscissae = concentration of urea in per cent.

That radiation changes have taken place is evidenced by color and smell and by the fact that the solutions precipitate when the urea is dialyzed out. As 30 to 35 per cent urea will denature fairly rapidly at 40° there will be urea denaturation as well as radiation denaturation, but as the results are the same if the urea is dialyzed out before heating to 40° C. it is probable that the radiation changes take place in the presence of urea and urea only prevents the flocculation of the denatured protein. If the concentration of egg albumin is reduced the opalescence is abolished by a lower concentration of urea. A 0.45 per cent albumin solution remained clear if radiated and heated in 30 per cent urea. The concentration of urea needed to prevent flocculation after radiation and moderate heat is proportional to the concentration of the protein (see Fig. 4).

If albumin solutions are radiated at pH 7.4 or 3.8 and heated to 40° C. for an hour they remain clear but precipitate on being brought to pH 4.8. This pre-

precipitation is prevented if 25 per cent urea is present but the solutions precipitate if the urea is dialyzed out.

Chick and Martin (11) found heat denaturation to be a reaction between protein and water with an extraordinarily high temperature coefficient. Heat coagulation involves two processes, the denaturation of the protein and the separation of the denatured protein in flocculated form.

The coagulation of proteins after exposure to ultraviolet radiation has been shown (9) to take place in three steps. Step 1 is a change produced by radiation shorter than λ 310 μ which takes place at any temperature and any pH. Step 2 is a heat change taking place in the light-denatured molecule with a high temperature coefficient which takes place at any pH and is effective at a temperature which does not produce any appreciable heat denaturation in the unradiated protein. Step 3 is the aggregation of the light- and heat-denatured molecules which occurs only near the isoelectric point.

One may summarize these changes as follows:

Denaturing agent	Steps in process of denaturation		
	(a)	(b)	(c)
Heat	—	Heat denaturation	Separation of denatured protein in flocculated form
	(a)	(b)	(c)
Ultraviolet radiation followed by moderate heat	Light denaturation	Heat change in light-denatured molecule	Separation in flocculated form

Urea in concentrations below 50 per cent (the concentration at which it splits the egg albumin molecule) appears to accelerate change (b) in heat denaturation although the precipitate formed is probably due to urea-denatured not to heat-denatured molecules. Above 50 per cent it prevents change (c) as the result of splitting the molecule.

In the case of radiation denaturation, steps (a) and (b) take place when egg albumin solutions are radiated and subsequently heated in the presence of urea. The third step, (c), of flocculation is prevented by 35 per cent urea if the solutions are radiated and heated at pH 4.8 and by 25 per cent urea if they are radiated outside the zone of opalescence. This is a concentration of urea which does not prevent the flocculation accompanying heat denaturation. As the concentration of urea needed to prevent step (c) in radiation denaturation is proportional to the concentration of protein, there is obviously a urea-protein complex formed which reacts to radiation and to heat in a way that differs from the protein molecule alone. The changes may be diagrammed as in Fig. 5. Al-

though for diagrammatic purposes the complex is shown as one molecule of protein plus one molecule of urea the relative concentration would suggest that the protein molecule may be enveloped in a shell of urea molecules. According to this interpretation the urea-protein complex will not aggregate producing flocculation, hence preventing step (c) in radiation denaturation, as the temperature of 40° C. used in radiation denaturation is not high enough to break down the complex. In heat denaturation, however, a temperature of 54° and over breaks down the urea-protein complex before a heat change *per se* takes place in the molecule and there is consequent flocculation of the urea-denatured molecule. When the molecules have been split by urea in concentrations of 50 per

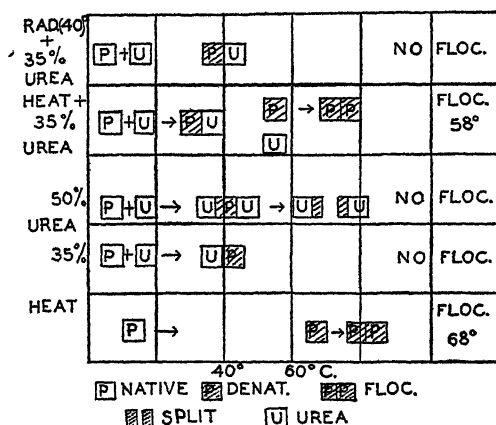


FIG. 5. Diagram showing interaction of heat, radiation, and urea on egg albumin molecule at different temperatures and concentrations of urea.

cent or over they are apparently no longer capable of flocculation until the urea is removed by dialysis.

Tyndall Beam Results.—An attempt was made to investigate the effect of ultraviolet radiation, heat, and urea on the shape of the albumin molecule by the depolarization of the Tyndall beam. The Tyndall beam from a solution in which the molecules are roughly spherical and small compared with the wave length of light is completely polarized. Depolarization is caused by increase in asymmetry or increase in size of the particles. The results were not satisfactory as the increase in size when opalescence developed at the isoelectric point completely masked the effect of change in shape. Opalescence less than 1.5 foot-candles was found to be determined more by asymmetry than by change in size. The results from the clear and slightly opalescent solutions are of doubtful significance, but are given in Table IV and indicate the following results.

1. The egg albumin molecule after radiation only has a fair degree of sym-

metry except at the isoelectric point and the same is true after radiation and heating to 40° C.

2. Asymmetry develops with aggregation which is prevented in the presence of urea.

3. Even when there is some degree of aggregation as evidenced by opalescence the presence of urea prevented any great degree of depolarization so that the protein-urea complex tends to remain a fairly symmetrical molecule.

TABLE IV
Depolarization of Tyndall Beam

Treatment	pH	Urea	Total opalescence	Bright component	Dark component	Depolarization
		<i>per cent</i>				<i>per cent</i>
Native albumin	4.8	—	0.07	0.13	0.05	37
Urea-denatured	4.8	35	0.2	0.4	0.10	25
	4.8	35	1.3	1.9	0.5	26
Radiation only	4.4	—	0.85	1.33	0.29	22
	4.8	—	0.14	0.245	0.1	40
	5.4	—	0.5	0.85	0.18	21
Radiation + urea	4.8	30	0.14	0.36	0.09	25
Radiation + heat 40° + urea	4.6	25	0.35	0.6	0.12	20
	5.0	25	1.0	1.7	0.34	20
	5.2	25	0.57	1.05	0.2	20
Heat 100° at pH 3.8 or 7.4 brought to different pH after heating	4.4	—	0.82	1.35	0.28	20
	4.8	—	1.75	2.5	1.0	40
	5.8	—	0.92	1.6	0.34	21
	5.0	25	2.2	3.4	1.28	37
	5.6	25	0.55	0.95	0.17	18

4. Heat denaturation at a pH outside the zone of opalescence leaves molecules fairly symmetrical until they are brought near the isoelectric point.

5. Urea does not prevent this asymmetry in heat-denatured molecules near the isoelectric point in 25 per cent concentration, which is probably due to the fact that heat breaks down the protein-urea complex.

These results tend to confirm the picture of the reaction of the protein molecule and of the protein-urea complex to radiation and heat that is given in Fig. 5.

Optical Rotation.—The change in optical rotation with heat denaturation previously observed (12) was confirmed and additional results obtained with

ultraviolet radiation and urea denaturation. Observations on undenatured albumin were made over a range of concentration and from pH 3.4 to 10.5. The results are given in Table V.

TABLE V
Optical Rotation of Egg Albumin. Effect of Denaturation and Changes in pH and Concentration

Condition	Concentration	pH	(Angle of rotation)	$[\alpha]_D^{22}$	Denatured
	<i>per cent</i>			<i>deg./dm.</i>	<i>per cent</i>
Fresh	1.2	4.8	-0.35°	-29.0	
	0.6	4.8	-0.34°	-28.0	
	0.3	4.8	-0.19°	-32.0	
	0.15	4.8	-0.10°	-33.0	
Fresh	1.2	3.4	-0.34°	-28.0	
		4.8	-0.34°	-28.0	
		6.0	-0.34°	-28.0	
		6.8	-0.33°	-27.5	
		7.4	-0.32°	-27.0	
		9.0	-0.33°	-27.5	
		10.5	-0.34°	-28.0	
Boiled 5 min.	1.2	3.4	-0.60°	-56.0	100
		6.4	-0.74°	-61.7	
		7.2	-0.61°	-50.8	
	0.6	7.0	-0.60°	-50.0	
Ultraviolet radiation					
20 min. (4° C.)	1.2	6.4	-0.39°	-32.5	35
1 hr. (4° C.)	1.2	6.4	-0.40°	-40.0	50
20 min. (4° C.)	0.6	7.0	-0.38°	-32.0	
40 min. (4° C.)	0.6	7.0	-0.50°	-42.0	
Urea 25 per cent	0.6	4.8	-0.33°	-27.5	
45 per cent	0.6	4.8	-0.34°	-28.0	
60 per cent	0.6	4.8	-0.43°	-36.0	

As has been previously observed (13) it was found that fresh undenatured albumin shows no variation in optical rotation between pH 3.4 and 10.5. There was evidence of a slight increase in optical rotation in a very dilute solution (0.15 per cent) but observations with such dilute solution are subject to large errors and the increase observed may not be significant. Boiling for 5 minutes at pH 3.4 or 6.4 \rightarrow 7.2 approximately doubled the optical rotation, the increase being greater, as pointed out by Barker (12) the closer the pH is to the isoelectric point.

When albumin is exposed to ultraviolet radiation at a temperature of 4° C. or

less, the first step in the denaturation process occurs, the coagulation which results from radiation being the result of a three-step process (9). The result of the first step is to increase the optical rotation and the increase is roughly proportional to the amount denatured as determined gravimetrically by subsequent coagulation of the light-denatured protein. Indeed a measure of the optical rotation of the radiated protein would serve as an excellent and very simple method for estimating the amount of denaturation following radiation for this particular protein, and shows a similarity here between radiation and heat denaturation.

Urea concentrations up to 45 per cent had no effect on optical rotation but there was a marked increase at 60 per cent urea. At higher concentrations it was impossible to obtain readings.

Where changes in optical rotation can be checked with ultracentrifuge determinations of molecular weight it is evident that association of molecules into larger complexes is accompanied by a decrease in optical rotation and dissociation by an increase. The stable values of optical rotation in all proteins in the pH range where the molecular size of proteins remains constant and the increase in optical rotation outside this range is one example of this. Association and dissociation, however, are not the only possible causes of change in the optical rotation values of a protein molecule.

Egg albumin denatured with 40 per cent urea has the normal molecular weight of 35,000 to 40,000 but it has been reported (8) that there is some dissociation of the molecule with concentrations of urea above 50 per cent. The change in optical rotation with urea above 50 per cent is probably accounted for by dissociation of the molecule.

The increase in optical rotation with heat and radiation denaturation, however, must be due to structural changes and not to change in size. No ultracentrifuge results for the molecular weight of egg albumin denatured outside the pH range where aggregation occurs are available. However, osmotic pressure measurements show no evidence of dissociation to account for the observed optical rotation changes.

One may conclude, therefore, that the egg albumin molecule shows an increase in optical rotation as the result of structural changes when denatured by radiation or heat. Urea denaturation does not affect the optical rotation of egg albumin, but splitting of the molecule by high concentrations of urea increases the optical rotation.

CONCLUSIONS

The extent of urea denaturation depends on the concentration of protein and urea and also on the temperature of the solution. Egg albumin solutions (0.9 per cent) are not denatured by 20 per cent urea, denature slowly with 25 per

cent urea, and denature rapidly with 35 per cent urea at room temperature. At a higher temperature 30 per cent urea is rapidly effective.

Denaturation of the egg albumin molecule by radiation or by heat is accompanied by structural changes as evidenced by optical rotation values, but is not accompanied by association or dissociation of the molecule in the pH range outside the zone in which aggregation follows denaturation.

Denaturation of the egg albumin molecule by urea produces no change in optical rotation until the concentration of urea is high enough to dissociate the molecule.

In the presence of urea a urea-protein complex is formed in which the protein is denatured but cannot flocculate because of the dispersive action of the urea. This prevents flocculation of proteins exposed to radiation and subsequent heating to 40° C. as the urea-protein complex is not broken down at a temperature of 40° C. The presence of urea therefore prevents the flocculation of proteins denatured by radiation.

The urea-protein complex is broken down by heating to 55–58° C. so that the molecules aggregate at a temperature below the temperature of rapid heat denaturation. This appears to be an acceleration of heat denaturation or a lowering of the heat denaturation temperature, but in reality is an effect of heat on the urea-protein complex which frees the urea-denatured protein and permits its aggregation.

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THE EFFECT OF TEMPERATURE ON THE RATE OF HYDROLYSIS OF TRIGLYCERIDES BY PANCREATIC LIPASE

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Few studies have been made concerning the effect of temperature on the rate of hydrolysis of triglycerides by lipase. Balls, Matlack, and Tucker (1937) reported that the triglycerides of fatty acids containing less than 8 carbon atoms were not split by pancreatic lipase any faster at 40° than at 20°C. and that the hydrolysis was remarkably rapid even at 0°C.

Shortly after the completion of the present investigation Sizer and Josephson (1942) published data on the effect of temperature on pancreatic lipase hydrolyzing excess tributyrin from -70° to 50°C. In accordance with the practice of Crozier (1926), they applied the Arrhenius equation:

$$\ln \frac{K_2}{K_1} = \frac{\mu}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

where μ represents the slope of the line drawn through the experimental points when log rate of the reaction is plotted against the reciprocal of the absolute temperature. The temperature characteristic (μ) is considered to be the energy of activation of the reaction expressed in calories per mol. These investigators found a μ of 7,600 for the enzyme acting from 0° to 50°C.

With the exception of this report the energy of activation of lipase has not been experimentally determined although a few attempts have been made to calculate the value from scattered data in the literature. For example, a $\mu = 16,700$ was computed (Waksman and Davison, 1926; Euler, 1920) from the work of Taylor (1906), in which the rate of hydrolysis of triacetin by castor bean lipase was compared at 18° and 28°C. Similarly, Lineweaver (1939) secured a $\mu = 4,200$ for the hydrolysis of ethyl butyrate by pancreatic lipase calculated from data of Kastle and Loevenhart (1900). A temperature characteristic of approximately 8,000 can be calculated for the same system, however, from the data in a report by Kastle, Johnston, and Elvove (1904). This value of 8,000 agrees well also with values computed from results of Terroine (1910) and Nicloux (1904). Since these temperature characteristics were computed from very meager data little significance can be given to them.

Method

The method suggested by Schwartz (1942) for the study of the hydrolysis of triglycerides was used with certain modifications. This procedure depended upon the

measurement of change in pH by the glass electrode due to the liberation of fatty acid. In the present work the reciprocal of the time required to hydrolyze a given fat to the same extent at the different temperatures was taken as a measure of the rate of reaction. A buffer of pH 9 consisting of K_2HPO_4 and KOH, an emulsifier of gum arabic, and a glycerol extract of lipase was used in all experiments. With dilute fat emulsions it was found that hydrolysis to a pH of approximately 8.1, for example, would take place at the higher temperatures in about 10 minutes. With greater concentrations of fat, however, this pH was reached in too short a time to enable convenient measurement, and consequently the hydrolysis was allowed to proceed to a lower more suitable pH. Preliminary investigations showed that this improved the accuracy of the results.

Inasmuch as the pH of a buffer is a function of the temperature the hydrolysis of a fat emulsion to the same pH at different temperatures would not represent the same degree of hydrolysis. Accordingly, the effect of temperature on the pH was determined for several buffers ranging from 8.1 to 6.5. These were prepared by adding

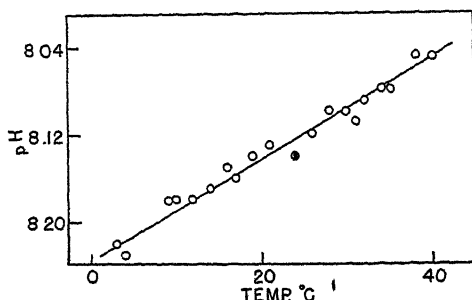


FIG. 1. The effect of temperature on the pH of a phosphate buffer (see text).

graded quantities of butyric acid to the original phosphate buffer. For each buffer the pH was directly proportional to the temperature over the short range studied, 2° to 40°C. (Fig. 1), although the slopes of the lines were different. Thus the enzymatic hydrolysis of a fat emulsion was allowed to proceed at a given temperature to that pH read from the curve plotting pH against temperature. The reciprocal of the time required for this to take place was recorded.

It is essential to compare the rates of hydrolysis of fat emulsions at the different temperatures when they are in the same degree of emulsification. Therefore, a stock emulsion was prepared, stored at a low temperature, and 20 ml. portions of it removed when needed. Esters which are hydrolyzed by water alone, such as tripropionin, were not studied because the amount of hydrolysis during the storage period and during the actual determination itself would be erroneously attributed to the enzyme. Triglycerides from butyric to caprylic inclusive were studied since they are not hydrolyzed by water to any measurable extent under the experimental conditions. The temperature of the emulsion was controlled to $\pm 0.05^\circ\text{C}$. by means of a water bath.

RESULTS

The effect of temperature on the rate of hydrolysis of triglycerides by pancreatic lipase was analyzed so that temperature characteristics could be meas-

ured for the reactions. Fig. 2 illustrates the data obtained for the hydrolysis of 0.05 M tributyrin by the enzyme for 36 temperatures between 2° and 37.2°C. Since the relationship between the reciprocal of the absolute temperature and log rate of hydrolysis is linear from 2° to 28°C., the data can be expressed by the Arrhenius equation. The value of μ was calculated to be $8,900 \pm 100$. Above 28°C. the relationship is no longer linear, probably because of thermal inactivation of the lipase. When only a few temperatures (10 to 12) were studied the μ for the same concentration of tributyrin was approximately $8,900 \pm 1,000$. It was believed, nevertheless, that the evaluation of μ from 10 to 12 determina-

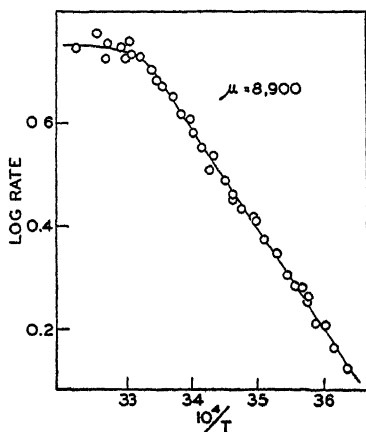


FIG. 2

FIG. 2. Log (rate of hydrolysis $\times 100$) of 0.05 M tributyrin by lipase plotted against $10^4/T$.

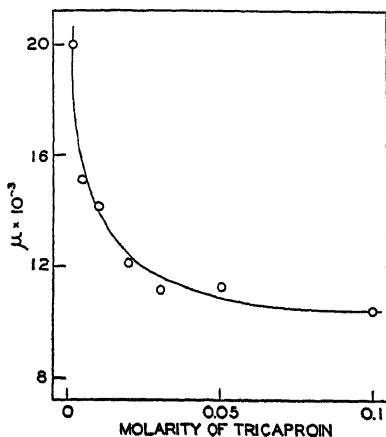


FIG. 3

FIG. 3. Relationship between the molarity of tricaproin and the temperature characteristic.

tions would be sufficiently accurate to establish the effect of variation of substrate concentration on the temperature characteristic.

The values determined in this way for various concentrations of several triglycerides are recorded in Table I. In the case of tributyrin, the mean of the μ values for all concentrations, excepting the value obtained with the most dilute concentration, was 8,500. This value compares favorably with that of 8,900 determined more accurately for one concentration. The temperature characteristic obtained for the most dilute emulsion of tributyrin, 10,200, is outside the experimental error of the mean and this deviation may be significant. The values found for the highest and lowest concentrations of trivalerin were 9,100 and 12,400 respectively, revealing a trend to higher values as the concentration decreases. The temperature characteristics for concentrations of tricaproin (0.1, 0.05, and 0.03) were constant (10,400 to 11,200). Further dilution resulted

in progressively larger values, 20,000 being obtained with 0.002 M concentration. Fig. 3 illustrates the curvilinear increase in μ upon dilution of the emulsion of caproin. This trend is emphasized further with heptylin. The highest concentration gave a low $\mu = 9,600$, while the value for the lowest concentration,

TABLE I
Variation of μ with Molarity of Triglyceride

Molarity	Butyrin	Valerin	Caproin	Heptylin	Caprylin
0.1	9,500	9,100	10,400	—	—
0.075	7,800	—	—	—	8,800
0.05	8,900	10,700	11,200	9,600	8,800
0.03	7,800	11,600	11,100	11,200	10,300
0.02	8,500	11,400	12,100	13,500	12,000
0.01	7,900	12,400	14,100	14,600	15,300
0.005	9,200	—	15,100	18,800	17,900
0.003	10,200	—	—	—	—
0.002	—	—	20,000	22,300	23,700

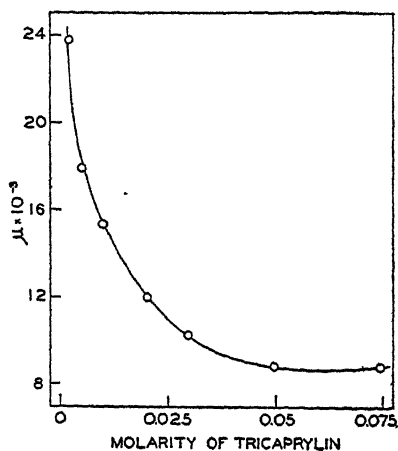


FIG. 4. Relationship between the molarity of tricaprylin and the temperature characteristic.

0.002 M, was 22,400. The μ for tricaprylin increased in a similar curvilinear manner from 8,800 with concentrated emulsions to a maximum value of 23,700 with 0.002 M (Fig. 4).

A given concentration of triglyceride gave a constant μ value regardless of the quantity of enzyme used or to what per cent hydrolysis the reaction was continued. In the hydrolysis of 0.02 M heptylin, for example, the same μ was obtained even when the degree of splitting was varied by 650 per cent.

DISCUSSION

Sizer and Josephson (1942) reported a temperature characteristic of 7,600 for the lipolytic hydrolysis of a concentrated tributyrin emulsion. This value compares well with the mean value of 8,500 for the hydrolysis of tributyrin by lipase obtained in this study. Although these determinations were made by two widely different methods, the values differ by only 900.

From data in the literature, temperature characteristics of 4,200 and 8,000 can be calculated for hydrolysis catalyzed by pancreatic lipase. The values obtained in this study varied from 7,800 to 23,700 and are characteristic of lipase acting on different substrates in a variety of concentrations in this particular heterogeneous medium.

Haldane (1930) has pointed out that if the dissociation constant of the compound formed between enzyme and substrate does not have a low temperature coefficient, the true energy of activation will not be determined in low substrate concentrations. He states, "Sub-maximal substrate concentrations should give a spuriously low or high temperature coefficient, the increase in the velocity of transformation of the enzyme-substrate compound being partly counteracted by the decreased formation of the compound or augmented by its increased formation." Thus it may be that the compound formation between glyceride and enzyme is endothermic and this results in increased μ values at low substrate concentrations.

It is possible too that the temperature characteristic of the reaction between enzyme and substrate differs for mono-, di-, and triglycerides. At the lower concentrations, particularly with the longer chained fats, a greater proportion of the insoluble di- and monoglycerides may be hydrolyzed. In a previous report Schwartz (1942) presented data indicating that more and more of the di- and monoglycerides are split by the enzyme as the length of the carbon chain increases.

SUMMARY

1. The temperature characteristics for the hydrolysis of various concentrations of tributyrin, trivalerin, tricaproin, triheptylin, and tricaprylin have been determined.

2. The μ values for the hydrolysis of all concentrations of tributyrin by pancreatic lipase, except the most dilute, were found to be constant within the experimental error, $8,500 \pm 1,000$.

3. The temperature characteristics for the hydrolysis of trivalerin, tricaproin, triheptylin, and tricaprylin varied from approximately $8,500 \pm 1,000$ for the high concentrations to 12,400, 20,000, 22,400, and 23,700 respectively for the most dilute concentration of each.

4. An interpretation of these results was presented.

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THEORY AND MEASUREMENT OF VISUAL MECHANISMS

IX. FLICKER RELATIONS WITHIN THE FOVEA

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I

In our examination of some essential propositions of visual theory it has been necessary to determine flicker recognition contours within the human fovea, with reference to light-time fraction (t_L) and wave-length composition as controlled parameters: It is also desirable to consider in this connection the area (and form) of the test patch as well. We give now an analysis of some of the phenomena encountered, although we do not for the present discuss area or form as variables within the fovea. We are to deal subsequently with conclusions arising from the study of reasonably homogeneous data on flicker recognition as a function of flash frequency and flash cycle form, flash intensity, image area and form, retinal location, wavelength composition of the light, and the fraction of the cycle-time occupied by light. It has been demonstrated¹ that the precise relation between flash frequency F and $\log I$ critical for recognition of flicker is defined by a contour of which the properties express the participating influences of quite a number of such variables. The present paper supplies additional data for the theory of flicker recognition in terms of the multivariate nature of visual response.

The character of the rôles of variables such as have been mentioned is, for the human observer, so far as exploration has gone, entirely consistent with the effects obtained on the corresponding contours for forced reactions to perceived moving stripes in a great variety of animals,² and is consequently non-specific. The basic analytical form of the F - $\log I$ contour is the same, a normal probability summation; and so also are the qualitative effects produced on its shape and position, in various animals, by altering the retinal receptive area, the light-time fraction, and the wavelength composition of the light, regardless of the type of eye concerned² ("camera" or "apposition"). The correlated changes in the specific $F - \log I$ contours, and in addition those produced by altering the temperature of the organism³ (which have not yet been tested with man), are directly in keeping with the general theory of the $F -$

¹ 1937-38, *J. Gen. Physiol.*, **21**, 203; 1940-41, **24**, 505, 635; 1941-42, **25**, 89, 293, 369.

² 1936-37, *J. Gen. Physiol.*, **20**, 393, 411; 1937-38, **21**, 17, 223, 318, 463; 1938-39, **22**, 311; 1938-39, **22**, 463, 487, 795; 1939-40, **23**, 143, 531; 1941-42, **25**, 381.

³ 1939-40, *J. Gen. Physiol.*, **23**, 143, 531; 1939, *Proc. Nat. Acad. Sc.*, **25**, 78.

log I contour which we have advocated.⁴ Quantitatively they support the idea that flicker recognition, as a forced response, is due to the summative action of elements of effect produced in a population of intrinsically fluctuating neural units. The properties of other analytically usable end-points for visual response are consistent with this.⁵ Among these properties not least significant are those of the *variation* of I_c , the flash intensity critical for flicker.¹ They give an objective standard of homogeneity of data⁶ and they also give a means of demonstrating the basically statistical nature of the critical intensity for single discriminatory responses.⁷

In the present observations several wavelength compositions of light have been used with a single small test area in the fovea, and at several light-time fractions. The resulting properties of the $F - \log I$ function are to be compared with those observed for other test areas and different retinal locations, for the same observer, having particular reference to the problem of visual integration. The dynamical nature of visual response cannot be interpreted simply in terms of the shape of the response contours under a fixed set of chosen conditions, because this shape is in general a function of these conditions; it is also a function of the observer and of the eye used; it is not invariant when these conditions are changed. This is likewise true for the relations between the contours for different parts of the spectrum with conditions otherwise the same.⁸ A guiding thought has been to recognize the respective parts taken by (a) number of neural units and (b) numbers of elements of effect⁹ produced in the determination of the visual effect used as end-point. It is in some important respects possible to improve the analytical conditions by working with simplex (flicker) performance contours, as obtained within the fovea.

II

The observational methods have been described previously, as well as the methods of computation.¹⁰ The same observers (W. J. C. and E. W.) were concerned

⁴ Cf. 1939-40, *J. Gen. Physiol.*, **23**, 531, etc.

⁵ 1940, *Proc. Nat. Acad. Sc.*, **26**, 54; 1939, *Science*, **90**, 405.

⁶ 1937, *Proc. Nat. Acad. Sc.*, **23**, 23; 1938, **24**, 130; 1939-40, *J. Gen. Physiol.*, **23**, 101, etc.

⁷ 1936, *Proc. Nat. Acad. Sc.*, **22**, 412; 1937, **23**, 23.

⁸ 1941-42, *J. Gen. Physiol.*, **25**, 293, 369, 381.

⁹ 1936-37, *J. Gen. Physiol.*, **20**, 393, 411; 1938-39, **22**, 311; 1938-39, **22**, 795; 1939-40, **23**, 531; 1940-41, **24**, 635; 1941-42, **25**, 89, 293, 369, and two following papers.

In a recent book, Bartley gives a figure purporting to show that this rule concerning the effect of the light-time fraction differs from that found by other investigators (Bartley, S. H., 1941, *Vision: A study of its basis*, New York, Van Nostrand Co., p. 122, Fig. 31). In fact, however, Bartley draws graphs on coordinates of F and t_L/t_D , and not of F_{max} . and $t_L/(t_L + t_D)$. We gave a fairly detailed account of the necessity for using the latter coordinates, in our first papers on the subject (1937-38, *J. Gen. Physiol.*, **21**, 313, 463).

¹⁰ 1940-41 *J. Gen. Physiol.*, **24**, 505, 635; 1941-42, **25**, 89, 293.

throughout. Monocular data (left eye) are discussed here. For the present experiments a square image measuring at the retina 0.602° on a side was provided by suitable adjustment of the slits in one limb of the visual discriminometer.¹⁰ A very small red dot immediately above this square image was formed by another beam of the instrument illuminating a minute hole in metal foil. By suitable adjustment of the intensity of this image it was seen only as red when within the fovea, and by practice it could be held at the lower margin of this area. This method of fixation is not perfect, but the properties of the data (including the variation of I_c) at the lowermost F range, where alone it was really needed at all, show none of the irregularities to be expected if this procedure had not insured adequate fixation.

When the plots (Figs. 1 and 2) are examined, certain quite minor but systematic departures are observed; thus the value of mean critical intensity I_m for the colored lights at $F = 2$ tend to be a little too high, while those at $F = 5, 8$, and 10 tend to be slightly too low (by the test of the adjusted probability integral for F vs. $\log I_m$). Minor departures of this kind have been found in other cases,¹¹ and have there been traced to a correlation with properties of particular filters ("neutral") used for the step-wise control of intensity levels. In the present instance they are likely due to the same causes, in part at least. The spectral transmissions by the filters used have been recorded and studied.

There is no discontinuity in the curves describing the data (Fig. 1) even though the fixation point be obliterated at $F = 10$ and above, and there is then no change in the scatter of the individual measurements of critical intensity for flicker (Fig. 3). This is important evidence, because at flash frequencies $F = 2$ to 8 or thereabouts the fovea adapted to the prevailing critical intensity for flicker fusion with this area of image can scarcely be said to give rise to the consciousness of light at all, although the incidence of flicker is detected with precision. There is no evidence that, under these conditions, even a very tiny illuminated dot quite nearby should influence the subjective end-point for flicker. This is especially clear since the curves for light-time fractions $t_L = 0.10$ and 0.90 are found to be quite parallel (with each F_{max} put = 100), as is shown in Fig. 3. It should be pointed out that there is nothing necessarily mysterious about the ability to detect flicker end-points at flash illuminations so low that the fused, non-flickering field is almost or indeed quite below the visual threshold; it is known that judgment of intensive differences can be made with lights not consciously perceived.¹²

III

The observations were planned as part of a larger scheme, now completed, involving the determination of flicker contours with several wavelength zones, with different light-dark proportions, and with a series of image areas on different portions of the retina. The tests are laborious and time-consuming. If the findings are to form a set of intercomparable data, steps must be taken to assure that no serious changes in an observer have occurred during the period concerned. These steps included as an important feature the systematic study of the variability of the observer's performance. The several sets of

¹¹ 1938-39, *J. Gen. Physiol.*, **22**, 311; 1940-41, **24**, 625; 1941-42, **25**, 381.

¹² Cf. Miller, E. G., 1939, *Am. J. Psychol.*, **52**, 562, etc.

measurements obtained were so far as possible designed to give information on the rôle of the variables mentioned with the smallest convenient effort. Thus, only two light-time fractions were used here, since it has been sufficiently well established that for simple test-fields (such as used here) F_{max} and τ' are rectilinear functions of $t_L/(t_L + t_D)$, while $\sigma'_{\log I}$ is invariant,⁹—unless subdivided test images are used with moving stripes¹³ producing flicker; the complications arising in these latter cases we discuss elsewhere.¹⁴ (F_{max} = the upper asymptote of the $F - \log I$ curve; τ' = the abscissa of inflection; $\sigma'_{\log I}$ = the standard deviation of its first derivative, with F_{max} put at 100.)

Visual performance contours for test areas entirely within the fovea are almost without exception simplex; they form single continuous curves, not the duplex functions characteristic of test areas in or including extra-foveal regions. One of several exceptions to this is seen in the relation of (extinction) threshold intensities to image area, within the fovea. (Another exception, which we shall later discuss at length, is found in the dependence of intensity critical for various different end-points upon the exposure-time.) As given, for example, by the data of Graham, Brown, and Mote, and Graham and Bartlett,¹⁵ the simple power function relating ΔI_0 to A changes exponent abruptly (white light, and red) at image areas of ca. 6 to 9 minutes diameter. This is paralleled by the behavior of similar data, including those not gotten by the extinction-of-brightness procedure, and for larger areas and in the periphery of the retina (*cf.* Crozier and Holway¹⁵), as is shown in detail in another place (Crozier¹⁵); reasons have been given¹⁵ for not necessarily regarding this change of slope as signifying structural duplexity. Generally, the properties of the simplex foveal contours have been taken to describe the properties of a purely cone receptor population. In comparing the parameters of such foveal flicker contours with those presumably for the cone units of extra-foveal regions, it has been found necessary to assume that the extra-foveal "cone" parameters for flicker are not influenced by concurrent rod excitation, although cone excitation does influence the neural results of rod excitation.¹⁶ In testing this notion, however, data are required for estimating the properties of the flicker contour parameters as a function of at least several variables, since the rôles of area, light-time fraction, and the like are quantitatively altered when the retinal region tested is changed.⁸ Consequently, comparisons of flicker excitability cannot have general theoretical significance if, for example, one has at hand merely curves for F vs. $\log I$ in different retinal locations with the same image area, light/dark ratio, and wavelength composition of light.

A basic clue to the systematic ordering of the properties of flicker contours is found in the consideration that the "elements of neural effect" responsible for the discrimi-

¹³ 1941-42, *J. Gen. Physiol.*, **25**, 369.

¹⁴ 1941-42, *J. Gen. Physiol.*, **25**, 369; and two following papers.

¹⁵ Graham, C. H., Brown, R. H., and Mote, F. A., 1939, *J. Exp. Physiol.*, **24**, 555. Graham, C. H., and Bartlett, N. R., 1939, *J. Exp. Psychol.*, **24**, 574. 1939-40, *J. Gen. Physiol.*, **23**, 101. The kinetics of adaptation, in preparation.

¹⁶ 1941-42, *J. Gen. Physiol.*, **25**, 89, 293, 369; 1941-41, **24**, 505, 635.

nation of flicker are produced by neural units which fluctuate in their capacities to produce neural effects.¹⁷ For a given retinal region $\sigma'_{\log I}$ is a function of the number of units involved, whereas F_{max} , and also τ' at constant temperature, depend on the mean numbers of elements of effect produced by such units.¹⁸ From this standpoint we shall consider the properties of the foveal flicker contours obtained with a white light and with certain color bands filtered from this white. The filters employed have already been used in various tests with a larger image in other retinal regions, and for other organisms.¹⁸

Violet, blue, green, and red lights were produced respectively by filters Corning 511, Wratten 47, 58, and 70. For some tests a special orange-yellow filter was used also. The intensity scale used in Figs. 1 and 2 is one of photometric brightness based on achromatic matches with the standard white; for certain other purposes a relative energy scale is used, based on matching with the standard white by using a vacuum thermopile with galvanometer as a null instrument.¹⁸

Measurements are collected in Tables I-V. Certain subsidiary measurements are referred to subsequently. Each I_m entry is the mean of ten consecutive readings, of which P.E.₁ indicates the dispersion. For each wavelength composition the observations were taken in several overlapping groups. No systematic drifts were detected, and (as shown subsequently) the variability of the critical intensities also retains a consistent character.

IV

Probability integrals adjusted to the data of Tables II-V are shown in Fig. 1. That a simple probability integral describes such foveal data over their whole range has already been shown, with the measurements for *white* and *blue* as examples.¹³ The curves differ slightly in F_{max} , and in $\sigma'_{\log I}$ (cf. Fig. 2), and they also differ in their positions on the $\log I$ axis (τ').

The general rules already noted^{9,18} are again confirmed: F_{max} declines as t_L is made greater; τ' increases; but $\sigma'_{\log I}$ is specific for each wavelength composition (at fixed image area). The image area is quite small here, and the change of F_{max} , in passing from $t_L = 0.10$ to 0.90 is less than for a larger area (6.13° square).¹⁹ But, as with the much larger square, the rate of change of the white (*W*) F_{max} , as a function of t_L is distinctly greater than for *V*, *B*, *G*, or *R*. For the latter, the change in the *V* F_{max} is greatest, and the changes in *G* and *R* are least. This order confirms that found with the larger area used.¹⁹ There is one difference, however: with a foveally centered square 6.13° on a side F_{max} for $t_L = 0.10$ was in the decreasing order *V*, *G*, *B*, *W*, *R*, whereas here with the square 0.6° on a side the order is *W*, *B*, *V*, *R*, *G*. The interesting fact that F_{max} can be higher for a colored light than for the white from which it is filtered is, however, again shown in the present data at $t_L = 0.90$, where the

¹⁷ Cf. footnotes 2, and 12 and 1936, *Proc. Nat. Acad. Sc.*, **22**, 412, 1940, **26**, 54, 334, 382.

¹⁸ Cf. 1941-42, *J. Gen. Physiol.*, **25**, 89, 293, 381.

¹⁹ 1941-42, *J. Gen. Physiol.*, **25**, 89, 293.

TABLE I

Data for flicker contours with "white" light, W. J. C., monocular (left eye), square image $0.602^\circ \times 0.602^\circ$, in the fovea; $t_L/(t_L + t_D) = 0.50$ and 0.90 . Each I_m is the mean of ten values, I_1 ; P.E.₁ is the dispersion of these ten; I_m = mean flash intensity, in millilamberts.

White

F per sec.	$t_L = 0.50$ $\log I_m$ $\log \text{P.E.}_1$	$t_L = 0.90$ $\log I_m$ $\log \text{P.E.}_1$
2		$\bar{4}.0233$ $\bar{6}.3971$
4		$\bar{4}.6901$ $\bar{5}.2030$
6	$\bar{4}.1870$ $\bar{6}.6839$	$\bar{3}.1208$ $\bar{5}.5228$
8	$\bar{4}.6125$ $\bar{6}.9542$	$\bar{3}.4646$ $\bar{5}.9376$
10	$\bar{4}.8739$ $\bar{5}.4003$	$\bar{3}.9681$ $\bar{4}.2958$
	$\bar{3}.0820$ $\bar{5}.3892$	$\bar{3}.7322$ $\bar{4}.4296$
	$\bar{3}.0730$ $\bar{5}.5443$	
12	$\bar{3}.1169$ $\bar{5}.5552$	
15	$\bar{3}.5047$ $\bar{5}.8568$	$\bar{2}.2526$ $\bar{4}.9085$
20	$\bar{3}.8919$ $\bar{4}.1956$	$\bar{2}.7221$ $\bar{3}.1713$
	$\bar{3}.9434$ $\bar{4}.4365$	$\bar{2}.7292$ $\bar{3}.1380$
25	$\bar{2}.5392$ $\bar{4}.8819$	$\bar{1}.2869$ $\bar{3}.8409$
	$\bar{2}.4390$ $\bar{4}.8530$	
30	$\bar{2}.8781$ $\bar{3}.3892$	$\bar{1}.7728$ $\bar{2}.2509$
	$\bar{2}.8871$ $\bar{4}.2774$	
35	$\bar{1}.2953$ $\bar{3}.7950$	0.2097 $\bar{2}.6973$
	$\bar{1}.3722$ $\bar{3}.7568$	
40	$\bar{1}.7455$ $\bar{2}.0639$	0.7526 $\bar{1}.1602$
	$\bar{1}.7547$ $\bar{2}.2431$	
	$\bar{1}.7705$ $\bar{2}.2655$	
43	0.2819 $\bar{2}.7266$	
45	0.6182 $\bar{2}.9669$	1.7122 0.2311
	0.6075 $\bar{1}.0783$	
	0.2960 $\bar{2}.8244$	
	0.8016 $\bar{1}.3600$	
48	1.2169 $\bar{1}.9516$	2.4344 0.9652
	1.2991 $\bar{1}.7701$	
50	2.2835 0.8726	3.2477 1.7208
	2.2667 0.9207	
	2.2831 0.9022	
51	3.0046 1.4475	
	2.6233 1.3101	
	3.5670 1.9652	

order is $B > V > R > W > G$; the differences are of course small, but it is clear that (as we have already emphasized¹⁹) comparisons cannot be made unambiguously unless contours are obtained over a range of values of each of the significant variables. For instance, the relative magnitudes of F_{max} , for the particular colored lights at a given t_L are really dependent on the different specific changes of F_{max} , as a function of t_L , image area, and retinal location.

The fact that, in Fig. 1, the several values of $F_{max.}$ are really so very similar makes it possible to study the change of τ' as a function of t_L almost independently of the influence on $F_{max.}$ When the temperature of an animal can be altered (which does not change $F_{max.}$) it was found³ that τ' is an interpretable function of the temperature, and the corresponding function for mean intensity flux can be at least qualitatively indicated for the present data. It is true that the procedure for work with lower animals, where the relations of the $F - \log I$ contour have been studied, involved illumination of the whole eye. It would be interesting to know if the apparent temperature characteristic for the position of the human $F - \log I$ curve depends on the size of the test-patch,

TABLE II

Conditions as in Table I, but violet light and $t_L/(t_L + t_D) = 0.10$ and 0.90 .

Violet

F per sec.	$t_L = 0.10$		$t_L = 0.90$	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2	6.0040	8.3071	5.5643	7.9818
5	6.6905	7.2610	4.2239	6.7316
10	5.5649	6.0103	3.0940	5.3953
15	4.2316	6.7745	3.7964	4.3296
20	4.7701	5.1152	2.3396	4.7900
25	3.2336	5.6454	2.8378	3.2892
30	3.7612	4.4302	1.2893	3.9005
35	2.2424	4.6415	1.8151	2.2842
40	2.7967	3.2260	0.3534	2.8196
45	1.5578	2.0748	1.1182	1.6836
48	0.1485	2.6221	1.9260	0.2192
50	1.5091	1.9363	3.0869	1.7002
51	2.6949	1.6287		

and on the retinal location. It might not be difficult to supply reasons leading to the expectation that this could be the case for ranges of area in which A and $F_{max.}$ are not in simple proportion and in which $\sigma'_{\log I}$ is a function of A , indicating inhomogeneity. But, disregarding this possible complication, we have pointed out that the temperature characteristic for the percentage light-time required to produce a constant value of τ' , with the same flash-intensity at different temperatures, must be the same as that for $1/I$ at any fixed F and fixed t_L fraction. Put in another way,²⁰ "for a given change $\Delta\tau'$ the necessary change in the dark-time percentage is a declining rectilinear function of the change in $1/T^\circ$ required to produce the same change in τ' when $F_{max.}$ is constant." The particular point which can then be examined in the present data is the relation of τ' to t_L in the case of light from different regions of the spectrum. The argument in view is more reasonable when $F_{max.}$ does not change greatly

²⁰ 1939-40, *J. Gen. Physiol.*, 23, 531 (p. 548).

with t_L , as in the present data; because then one is not concerned with the secondary correlations possible between $F_{max.}$ and τ' . But a still different complication remains, namely that the test must ideally be restricted to cases in which the number of units concerned is the same, as would be indicated by the constant value of $\sigma'_{\log I}$.

TABLE III

Conditions as in Table I, but blue light and $t_L/(t_L + t_D) = 0.10$ and 0.90 .

Blue

F per sec.	$t_L = 0.10$ $\log I_m$ $\log P.E._1$		$t_L = 0.90$ $\log I_m$ $\log P.E._1$	
2	8.8261	9.2943	6.1911	8.7038
			6.2111	8.7243
4	7.3796	9.9040	6.8084	7.3294
5	7.5229	9.8340	5.0734	7.6386
8	6.1086	8.7360	5.5914	6.1383
10	6.4168	8.9361	5.9418	6.3762
12	6.7653	7.3316	4.2213	6.3394
15	5.0433	7.4782	4.5878	5.0066
18	5.4028	7.9671	4.9447	5.5579
20	5.6465	6.1416	3.1674	5.7170
22	5.8260	6.3006	3.3996	4.0671
25	4.1329	6.5779	3.6609	4.0649
28	4.3788	6.9027	3.9461	4.3361
30	4.5747	5.0881	2.1061	4.5873
33	4.8925	5.5004	2.4199	4.9361
35	3.0786	5.3460	2.6005	4.9413
38	3.4191	5.8582	2.9256	3.1361
40	3.6883	4.2569	1.1790	3.5737
43	2.1072	4.5549	1.6434	2.0733
45	2.4260	4.8995	0.0665	2.4652
			1.9334	2.3325
47	2.9831	3.5711	0.4917	2.9403
48			0.5279	1.0808
			0.7922	1.2781
49	1.7331	2.2983	1.2264	1.6974
50	0.3139	2.7549	1.8513	0.3232
51	1.3813	1.8253		

The argument is, briefly, that if the change $\Delta\tau'$ for a given change Δt_L is the same for different spectral regions, then temperature characteristics for the flicker contours obtained for these lights of differing wavelength composition should be the same, as already indicated—provided $\sigma'_{\log I}$ were the same for the different spectral regions. If the rate of change of τ' for change of t_L should be the same for V , B , G , R , and W , one could then conclude that the chemical mechanism governing the production of flicker recognition elements

is controlled in the same manner. In the data of the present experiment it is clear that the calculated $\Delta\tau'$ between $t_L = 0.10$ and 0.90 is largest for W , smallest for R , and very nearly the same for V and B . The values are:

$$(W - \Delta\tau' = 1.72)$$

V	1.49
B	1.51
G	1.56
R	1.45

There is thus a close parallelism between $\Delta\tau'$ for the several spectral regions and white and the respective values of $\sigma'_{\log I}$ (Fig. 2). From the inverse order

TABLE IV

Conditions as in Table I, but green light and $t_L/(t_L + t_D) = 0.10$ and 0.90 .

Green

F per sec.	$\log I_m$ $t_L = 0.10$ $\log P.E._1$	$\log I_m$ $t_L = 0.90$ $\log P.E._1$
2	$\bar{7}.6293$ $\bar{8}.0600$	$\bar{5}.1730$ $\bar{7}.5697$
5	$\bar{6}.3404$ $\bar{8}.6912$	$\bar{5}.8771$ $\bar{6}.3849$
10	$\bar{5}.1988$ $\bar{7}.6877$	$\bar{4}.7282$ $\bar{5}.2678$
15	$\bar{5}.8286$ $\bar{6}.3444$	$\bar{3}.3977$ $\bar{5}.9163$
20	$\bar{4}.4143$ $\bar{6}.8957$	$\bar{3}.9312$ $\bar{4}.4444$
25	$\bar{4}.9302$ $\bar{5}.2787$	$\bar{2}.4464$ $\bar{3}.1081$
30	$\bar{3}.3676$ $\bar{5}.8836$	$\bar{2}.9102$ $\bar{3}.2590$
35	$\bar{3}.8839$ $\bar{4}.4824$	$\bar{1}.4204$ $\bar{3}.9409$
40	$\bar{2}.4376$ $\bar{3}.0052$	$\bar{1}.9762$ $\bar{2}.4541$
45	$\bar{1}.2095$ $\bar{3}.8190$	0.7499 $\bar{1}.2878$
48	0.1557 $\bar{2}.4833$	1.6185 $\bar{1}.7951$
50	1.1384 $\bar{1}.3652$	2.7144 1.3601
51	2.2895 0.6951	

of the latter quantities it is deduced that the relative numbers of neural units concerned are

$$W > G > V, B > R$$

This is consistent with the effect of simply enlarging the area of the test-patch, as then $\sigma'_{\log I}$ and $\Delta\tau'$ are again inversely related.²¹

The behavior of $F_{max.}$ indicates no real correspondence with that of either $\Delta\tau'$ or $\sigma'_{\log I}$, the order being $W > B > V, R > G$. $F_{max.}$ is a measure of the total number of elements of effect obtainable and this quantity is determined by two independently varying things: the number of units concerned, and the mean frequency of elements of effect contributed by each. It is already known that $F_{max.}$ and $\sigma'_{\log I}$ can be caused to vary independently.⁹

²¹ Cf. paper on area vs. $\Delta\tau'$ and $\sigma'_{\log I}$ in preparation.

The inference is thus permitted that if the numbers of *units* could be made the same, the values of $\Delta\tau'$ for $t_L = 0.10, 0.90$ at different wavelengths would not differ significantly. (Note that $\sigma'_{\log I}$ for B and V do not really differ and that $\Delta\tau'$ is essentially the same for each.) The observed values of $\Delta\tau'$ do not depart significantly from rectilinear proportion to $1/\sigma'_{\log I}$. Thus there is no reason in these data to question the proposition that the chemical mech-

TABLE V

Conditions as in Table I, but red light and $t_L/(t_L + t_D) = 0.10$ and 0.90 .

Red

F per sec.	$t_L = 0.10$ $\log I_m$ $\log P.E._1$		$t_L = 0.90$ $\log I_m$ $\log P.E._1$	
2	7.9890	8.4614	5.5054	6.0261
4	6.6105	7.1335	4.1344	6.5511
5	6.8601	7.2780	4.3971	6.8684
8	5.4310	6.0119	4.9300	5.4489
10	5.7366	6.2613	3.2509	5.7145
12	5.9965	6.4227		
15	4.3930	6.8373	3.9168	4.4054
18	4.8054	5.3459		
20	3.0346	5.5870	2.5231	3.0742
22	3.2277	5.6903		
25	3.7245	4.1840	1.0373	3.3739
	3.5765	4.1324		
28	3.8896	4.5258		
30	2.1016	4.6297	1.5680	2.1563
	2.0906	4.4727		
33	2.3476	4.7675		
35	2.4999	3.1089	0.0407	2.6219
38	2.8581	3.3258		
40	1.0852	3.4245	0.6419	1.1103
43	1.5434	2.0155	1.0934	1.6001
45	0.0078	2.5243	1.5511	1.9914
47	0.4869	1.0395		
48	0.7803	1.0778		
49	1.2210	1.6881		

anism of excitation is the same for different spectral regions, although the shape of the flicker contour is definitely not the same for different retinal regions, sizes of image, or spectral compositions.

It has been attempted to deduce the kinetics of primary photic excitation from the *shape* of the performance contour, as in the case of flicker,²² part of the argument resting on the asserted invariance of the shape of this curve in terms of a 2-parameter function. It is now pointed out that (as in other cases²³) 3

²² Cf. Hecht, S., 1937, *Physiol. Rev.*, 17, 239.

²³ 1940, *Proc. Nat. Acad. Sc.*, 26, 54, 334, 382.

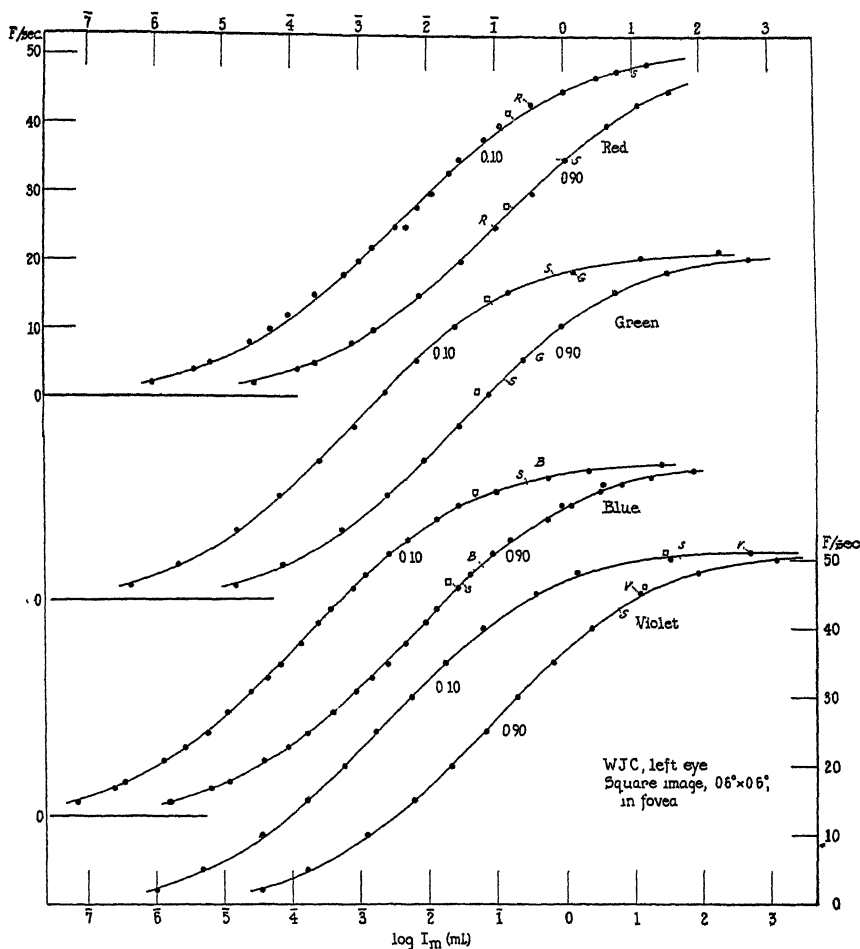


FIG. 1. Flicker response contours within the fovea; square image subtending 0.602° on a side, left eye, W. J. C.; for violet, blue, green, red; with light-time cycle fractions $t_L = 0.10$ and 0.90 . Data in Tables II, III, IV, and V. At the points on the curves marked by the specific symbol (V, B, G, R) the proper color is apparent. At the points marked S the critically fused field is smooth. At the points marked by a square, the outline of the test patch is clearly visible at critical fusion. The curves drawn are normal probability integrals (compare Fig. 2).

parameters are required, as with the probability formulation, but that this in fact does not lead to a conclusion that the simple basic physicochemical control of the production of elements of sensory effect is a complex function of area, retinal region, observer, eye, or wavelength. So far as concerns the deduction of the physicochemical primary properties of excitability, it is required to first dissect out those observable features of the measurements of responses mediated

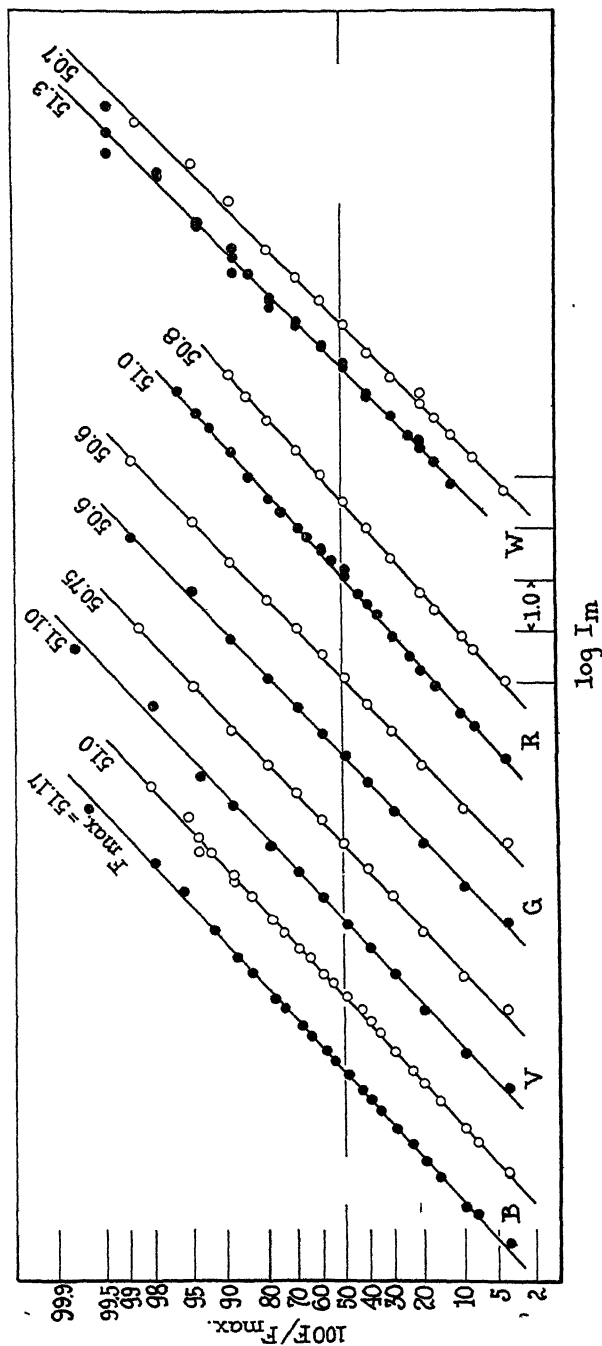


FIG. 2. The data of Fig. 1, and in addition data on white (W) for Table I, put on a probability grid. The respective values of the asymptotic upper maximum F are indicated. The pairs of curves are displaced laterally for convenience; the $\log I$ separation in each pair of curves is correct. In each pair the data for $t_L = 0.10$ (or for W , $t_L = 0.50$) are shown as solid dots, the data for $t_L = 0.90$ as open circles. The parameters of these functions are discussed in the text.

by this excitability which arise from the fact that the retinal mosaic and the visual system as a whole are a complex of cellular units.

As a consequence of this situation it is clearly necessary, for a determination of a valid spectral "visibility curve" for the flicker sensory effect as a function of wavelength, to obtain measurements of effect as a function of t_L and of stimulated area under conditions such that the values of $\sigma'_{\log I}$ and of F_{max} can be found the same for different wavelengths. Entirely analogous conditions hold for the use of neural end-points other than recognition of flicker. This is sufficiently indicated by the data on visual intensity thresholds as a function of λ when A and exposure time are varied.²⁴ It has been clear from the flicker data already described that with A constant and F_{max} made the same by suitably changing t_L , the correlated dependence of τ' for different spectral zones is not the same for different retinal regions. This could perhaps be explained on the basis of differential pigmentation (e.g., the presence of macular pigment), and in our data on a bird certain differences as compared with man have been successfully accounted for on this basis.⁸ These comparisons have been by way of the properties of τ' as a function of λ with F_{max} constant or nearly so. They thus involve the brightness or the mean energy required to activate one-half the potentially excitable flicker elements of fixed number and ignore the differences of $\sigma'_{\log I}$ for different λ -zones. As already pointed out here, this does not enable us to get very far in disentangling the questions concerned with the effects of λ on (a) numbers of units and (b) numbers or frequencies of elements of effect from each unit.

Using a foveally centered 6.13° square (observer, apparatus, and filters being the same as for the present paper) we found⁸ that $\sigma'_{\log I}$ ("cones") for white was intermediate, the numbers of cone units for the several kinds of light as deduced from the (inverse) order of $\sigma'_{\log I}$ being: $R > W > B, G > V$; for the present series the corresponding seriation is: $W > G > V > B > R$ (0.603° square). Thus, as regards *numbers of units* affected for a given potential magnitude of sensory effect in relation to discrimination of flicker, W was intermediate with the larger square but is clearly greater for the very small square. We can also compare with these seriations the less complete results independently obtained in another, extrafoveal, region of the retina,⁸ $B > W > R$ (6.13° square).

Only if the effectiveness of the *white* is regarded as some kind of synthesis of competing effects of spectral regions can such differences be understood.⁸ It cannot be decided without further investigation to what extent other visual data must also be regarded from this standpoint, but it is certainly legitimate to consider that the phenomena of flicker recognition give a proper basis for preliminary considerations. In terms of the numbers of neural units excited, and their retinal locations, white light may be more effective than R or B , or it may be intermediate between them. In terms of the total sensory effect ob-

²⁴ Results to be presented elsewhere.

tained it may be intermediate, or greater than R or B , and the relative efficiencies of R and B can be quite reversed. It is obvious that in comparing the several retinal parts here concerned the mere presence of the yellow macular pigment cannot wholly account for the results. It is true that certain parallelisms can be indicated in terms of visual thresholds. Thus at the fovea ΔI_0 , the dark adapted threshold intensity, is in energy terms least for lights from the G region of the spectrum and increases for B and R in that order, corresponding to the respective numbers of foveal neural units here deduced from the flicker data,²⁵ and the curves of ΔI_0 as a function of distance from the fovea do show crossings corresponding to the reversals of R and B in flicker.²⁵ These properties of ΔI_0 are also functions of A and exposure time, however, and no real analysis can be made until these functions have been explored.²⁴

In terms of the intensities required to activate half the potentially excitable elements of ("cone") flicker effect, with $F_{max.}$ made the same by altering t_L , it was found⁸ that W was intermediate in the case of the foveally centered 6.13° square, just as was the slope constant $\sigma'_{\log I}$. In the present data with the 0.603° square, τ' for W is higher than for any of the colored lights, in keeping with its smaller value of $\sigma'_{\log I}$ and consequently larger number of excited units. The values of τ' (photometric) at $t_L = 0.90$, for example, where the values of $F_{max.}$ are all very nearly the same, are

$W, \tau' =$	1.320 (ml.)
V	2.820
B	3.683
G	2.462
R	1.040

The corresponding values simply for $t_L = 0.10$ with the larger foveally centered (6.13°) square, no adjustment being made for $F_{max.}$, are in the order $B < V < G < R < W$, exactly as in the present data, while⁸ for the same square centered 6.4° on the temporal side of the fovea the values of τ' were also in the order $B < R < W$, just as for the present series.

Series of measurements with another observer (E. W.) show that, for the same small image area, left fovea, the "yellow spot effect" is much more marked. At $t_L = 0.50$ the curves are all steeper than for W. J. C., $F_{max.}$ is higher, and the values of τ' are higher. The interpretation of the rôle of this effect in flicker is complex, but what we have chiefly in mind here is displayed in the relative positions of the B and R curves. For the data on W. J. C. the τ' separation of B and R is $1.38 \log I$ units, the B curve τ' being smaller. For E. W. the R curve is 1.19 units below the B . Tests were also made with a special yellow-orange filter. In each case the Y and W curves are close together. For E. W. the Y curve is situated at the lowest intensities, for W. J. C.

²⁵ Cf. Wentworth, H. W., *Psychol. Monogr.*, 40, 1930; and footnote 21.

the B . Yet for retinal regions 6° to 10° off the fovea, or for images 3° square at the fovea, the order of effectiveness with each observer is just that already mentioned for W. J. C., r' being in the sequence $B < Y < R$ and agreeing closely in the absolute values for the two.

When the values of ("cone") r' are considered on the basis of relative energy, by way of the thermopile measurements, W with the 6° square was found intermediate when t_L was adjusted to make F_{max} , the same, but at $t_L = 0.90$ it is higher than for any of the spectral zones tested; this is also true of the present measurements with the 0.6° square, just as for the zebra finch.⁸ By this test R and W are *relatively* more effective for flicker, within the fovea alone as compared with the larger field, than are G , B , and V , in that order.

In making this comparison, as for others, it has to be kept in mind just what it is that one is testing. The frequencies of neural effects contributed by each unit at the point of flicker recognition can vary independently of the number of units excited, as is shown by the rôles of t_L , A , λ , and retinal position. Presumably the recognition of flicker is immediately due to the total number of the acting effects; but the circumstances of stimulation determine the number of units producing these effects and the intensity required to activate them. This may be seen in the comparison of the data for the 0.6° and the 6° squares foveally centered. By the $\sigma'_{\log I}$ test, the larger square involves a larger number of "cone" units, and as indicated by F_{max} , a larger total number of elements of effect. This is true for all the wavelength compositions, although the changes in $\sigma'_{\log I}$ are greater in the sequence $R > G > V > B > W$; yet the change in t_L required to give the same change in F_{max} , for example, is obviously much greater for the smaller area.

V

The variation of I_1 is of the same general order as that previously found for the same observer in similar experiments.¹ For a 6.13° square, foveally centered, with white light, $P.E._1/I_m$ was 0.0340 ± 0.005 , independent of intensity, and λ , and t_L . In the present data it is 0.0317 ; this corresponds to a mean precision of *ca.* 1 per cent in I_m . There is evidence that the difference between the two series, although small, may be significant, and is correlated with the lower levels of F_{max} , and of the numbers of units, in the present series; it probably cannot be exclusively a function of F_{max} , as the data on colored lights have already shown.¹

When dealing with samples of uncorrelated data from the same unrestricted universe, elementary statistical theory indicates that the scatter of σ_1 (or of σ_m for equivalent random repeated samples of the same size n) is given by $\sigma_\sigma = k\bar{\sigma}/\sqrt{2}$, where $\bar{\sigma}$ is the average of the standard deviations of the samples. But if by reason of internal correlations among the data, due for example to the lawful intrinsic fluctuation of the mechanism responsible for the data, and

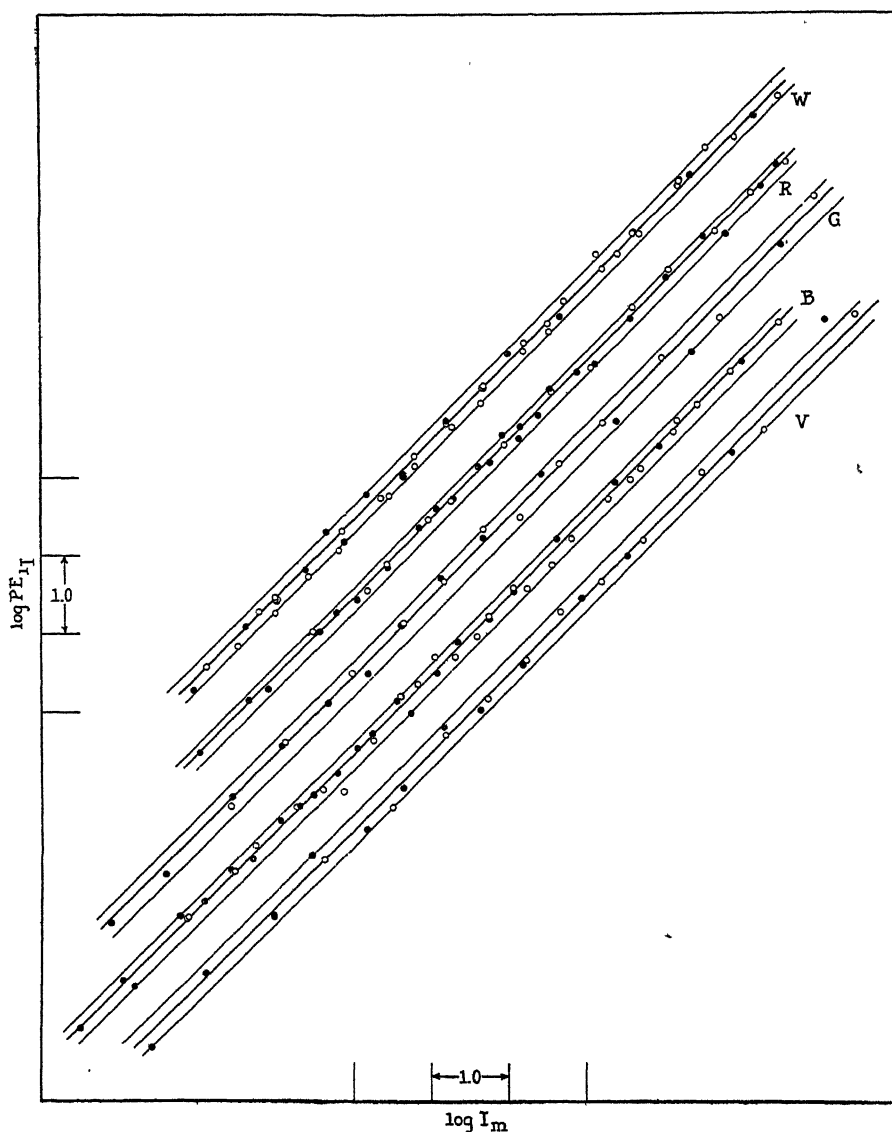


FIG. 3. On a double log grid the relationship between $P.E._{1f}$ and I_m is shown for the data of Tables I to V. The plots are displaced vertically for convenience. The intercept of the central lines which bisect each series of points is not the same. The mean value of σ_m is 1.46 per cent of I_m , and not > 2.6 per cent anywhere. The scatter of the points about the central lines is discussed in the text.

when this same mechanism is repeatedly tested, then it is also known that we must correct for this by including a measure of the intrinsic control of scatter. This correction takes the form

$$\sigma_{\sigma} = \bar{\sigma} \sqrt{1 - r} / \sqrt{2}.$$

In sufficiently homogeneous data, on flicker for example, it has been shown empirically that the *scatter* of the variation indices (σ , or P.E.) in plots such as those in Fig. 3, is not invariant. Thus, it is a function of F_{max} . when t_L is varied.¹ It is reduced, together with σ_1 , by binocular stimulation.¹ It is also a function of λ when t_L is constant,¹ although σ_1 is not. As the total size of the potentially excitable group of ("cone") units is increased, the precision with which σ_1 is exhibited is definitely diminished.¹ In the present case, we can roughly test this visually by comparing the breadth of the scatter bands in Fig. 3, which indicate the relative magnitudes of σ_{σ} independently of the intensity level. The mean values of P.E.₁/ I_m , and the scatter of this ratio, can of course be calculated directly.⁷ For the four color bands, where F_{max} is very nearly the same and changes so little with t_L , we find that these breadths are in the order $G > V > B > R$, which is precisely the order already deduced for the numbers of neural units excited. When, however, the number of units ($\sigma'_{\log I}$) remains the same but the mean frequencies with which they contribute are caused to change, we also find that the breadth of the scatter band increases as F_{max} increases.¹

It is clear that this can be developed as an independent test of the degree of internal correlation in the data. An important instance has to do with the measure of neural integration ("interaction") as affected by changes of retinal excited area, location, and form, which will be considered subsequently. Since enlargement of image area increases the ("cone") value of $1/\sigma'_{\log I}$, and the relation between A and F_{max} is not simple, experimental conditions must again be found for the analytical separation of these two factors. But this appears to be possible, and can obviously lead to an unequivocal objective index of visual integration. The properties of the variation of I_1 plainly show that the total number of units and of elements potentially excitable are concerned in the discrimination of flicker at all levels of F for any one contour. This is clearly consistent with the central nervous control of the recognition point, rather than with its determination by a progressively larger number of excited retinal units as F is increased. The index of integration supplied by the behavior of σ_{σ} is thus independent of the intensity level, and of the level of light adaptation to which the observer is adjusted along the flicker contour.

VI

The various properties of the $F - \log I$ contour are independent of subjective color. There are no discontinuities of the curve nor any changes in the varia-

tion of I_1 which could be correlated with perception of color. When a flickered square test-patch 6.13° on a side was used with various spectral bands a distinct "rod" component was present in the duplex contour. Color appeared at a rather high flash frequency and consequently at a high flash intensity along the fusion contour. It might conceivably have been argued that concurrent rod excitation could in some fashion be responsible for the high color threshold, although the effects of varying the light-time fraction¹⁹ would make this peculiarly difficult to understand. The log I separation of the approximate color thresholds on the different t_L curves for each color was such as to show that the color threshold along the contour for critical fusion is for each color governed by the mean intensity flux.

With simplex, foveal flicker contours the case may be considered without reference to possible "rod" complications. The observations show that even though only the foveal area is involved the color thresholds at flicker fusion are located at comparatively high flash frequencies (and flash intensities). One problem arising here is of course related to the often debated matter of the "photochromatic" or "achromatic" interval. It has been held for the foveal center that in the extreme red this interval between achromatic and chromatic thresholds is so small that it does not occur (as is true with some observers, but emphatically not for others). It is a simple matter, however, to show that even at the fovea this interval is a function of image area and of exposure time.²⁴ As ordinarily tested for, the foveal achromatic interval with our red is very small (0.01 log unit) for E. W., much larger for W. J. C., over exposure times for single flashes ranging from 0.0003 second to 0.5 second. Even with E. W., however, it is not until a comparatively high flash intensity is reached ($\log I$ (m.) = 2.53 for $t_L = 0.50$) that the red color is detected, corresponding to an achromatic interval of *ca.* 1.5 log I units along the flicker contour (for W. J. C., 1.20 is color threshold for this red, an achromatic interval of *ca.* 4 log units).

At the fovea the general order of (energy) intensities for the threshold of color is $R > V > B > G$ (dark adapted), or $R > V > G > B$ (light adapted). For our color bands the color thresholds appear along the flicker contours (W. J. C., $t_L = 0.90$) in the order of intensities (photometric)

$$\begin{array}{ll} R > G > V > B & \text{for the } 6.13^\circ \text{ square, and} \\ V > G > R > B > Y & \text{for the } 0.602^\circ \text{ square.} \end{array}$$

For the data on E. W. the order (0.6° square) is: $V > G > B > Y > R$; here the R color threshold is lower than for W. J. C., the G , B , and Y higher, V about the same. It is to be remembered that a factor in the incidence of the color threshold may well be the fusion of a direct color impression due to flashes of light with the after-image color impressions produced in the dark intervals; as we have earlier noted, these complementary color impressions can be obtained just below the proper color threshold at fusion.¹⁸

The sequence of subjective effects obtained as one goes up the $F - \log I$ fusion contour has been described for larger test-fields.¹ It is of some importance that on the present simplex contours precisely the same general sequence is obtained as F is increased: (1) the critically fused field is gray-blue; (2) it is granular or speckled; (3) it is "frosted"; (4) the outline of the square is clearly seen, and the field is smooth; (5) color is apparent. The order of this sequence, some features of which have been indicated in the graphs of Fig. 1, is not fixed; with R , for example, (5) occurs at lower fusion intensities than (4). The general point is to be made that this succession of phenomena cannot possibly be employed as diagnostic for the entrance of cone function in duplex contours.⁹ Nor can it be alleged that the well known scotopic bluish gray effect is due exclusively to excitation of rods.

The critically fused intensity level at which the *outline* of the square test-image is just perceptible may be taken as an index of visual acuity. It was recorded previously¹⁸ that while the color points on the $F - \log I_m$ curves at different values of t_L fall pretty closely at the same average intensity flux, for each color, the points at which the fused field becomes smooth and uniform tend on the whole to fall at about the same *flash* intensity. This is more definite when the criterion of clear vision of the margins of the foveal square is used. In a general way it is known that visual acuity (steady light) is said to decrease in the order $W < G < R < B < V$. The brightness of flashes producing discrimination of the square boundary, on the flicker fusion contour, ranged in the present experiments from $\log I_m = 2.65$ (W) to 1.00 (V) in the sequence of effectiveness $(1/I_m) = W > B > G > R > V$. In view of the quite different criteria of acuity implied, no complete parallelisms could be expected; this will be more fully examined in a following paper.

VII

SUMMARY

Flicker response contours (F vs. $\log I_m$) for a square image subtending 0.602° on a side, located in the fovea, are simplex probability integrals for a "white" and for four (five) spectral regions filtered from this white, and with different light-time fractions in the flash cycle. The subjective phenomena (the appearance of the field, the intensity threshold for color, and others) at the fusion points along these contours parallel in a variety of ways those obtained on duplex flicker contours resulting from the use of larger or eccentrically placed flickered images. These phenomena therefore cannot be held to indicate involvements of "rod" excitation.

The scatter of the index of variation of I_1 is such as to demonstrate the full participation of all the potentially excitable neural units at all levels of flash frequency, for each kind of light. The magnitude of this scatter, a measure of neural integration in visual performance, is a function of the *number* of these

units (with F_{max} , nearly constant); the two quantities vary together when wavelength composition of light is altered.

The properties of the contours for a white light and for the spectral regions filtered from it show that, for the image within the fovea, different numbers of units are excitable in flicker recognition according to the wavelength band used, and different mean frequencies of elements of effect under fixed conditions. The changes in the mean intensity for activation of these units as a function of the light-time fraction in the flash cycle are correlated with the numbers of these units; when this is corrected for, it is pointed out that despite the differences in shape of F vs. $\log I$ it cannot be concluded that the mechanism of excitation differs for different wave-lengths. It is indicated that "white" must be regarded as a synthesis, not a mere summation, of effects due to different spectral regions. Certain differences are pointed to as between foveal and more peripheral regions tested, and as between observers differing in the degree of the "yellow spot effect," with regard to the relative effects of wavelength and of image area. A general consequence is the outlining of conditions required for the precise comparison of excitabilities as a function of wavelength in the multivariate visual system.

STUDIES OF THE INNER AND OUTER PROTOPLASMIC SURFACES OF LARGE PLANT CELLS

I. PLASMOLYSIS DUE TO SALTS

By W. J. V. OSTERHOUT

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and The Marine Biological Laboratory, Woods Hole)

(Received for publication, August 1, 1943)

Electrical studies on *Nitella*¹ show that the outer, non-aqueous, protoplasmic surface (*X*) differs from the inner (*Y*). If cell sap is placed outside the cell so as to set up the chain

	<i>X</i>	<i>W</i>	<i>Y</i>	
Sap		Aqueous		Sap
outside		protoplasm		in vacuole

we find² a P.D. of about 16 mv. This would be impossible if the outer and inner protoplasmic surfaces were identical in their properties.

Further evidence of unlikeness is found in plasmolytic experiments. When cells are placed³ in suitably diluted sea water salts appear to penetrate the outer non-aqueous surface, *X*, more rapidly than the inner, *Y*. This increases the salt content and consequently the osmotic pressure of the aqueous layer, *W*, of the protoplasm so that it withdraws water from the vacuole and increases in thickness⁴ (Figs. 1 to 3). Thus the distance between *X* and *Y* may become very much greater than in the normal state.

In most cases some separation of *X* and *Y* takes place within a few minutes and the cell returns in an equal or shorter length of time to its original state when replaced in tap water, if the alteration has not proceeded too far.

As the process continues the shrinkage of the vacuole may produce a long narrow vacuole (Fig. 2) or it may break up into several spherical or elongated vacuoles (Fig. 3).

¹ *Nitella flexilis*, Ag.

² Cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, **11**, 391. The electrode in contact with *X* is negative in the external circuit to the electrode in contact with *Y*.

³ Sea water 6 parts plus tap water 1 part is suitable in many cases.

⁴ The cells, after being freed from neighboring cells, stood in the laboratory at 15°C. ± 1°C. in Solution A (cf. Osterhout, W. J. V. and Hill, S. E., *J. Gen. Physiol.*, 1933-34, **17**, 87) for several days. They belonged to Lot B (cf. Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sc.*, 1938, **24**, 312).

Temperature 25-30°C.

Dead cells do not give these results.

In some cases the outer surface, *X*, shrinks away from the cell wall. Its position is easily seen as the chloroplasts adhere to it and come away with it when it shrinks away from the cellulose wall.

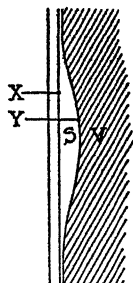


FIG. 1. The beginning of separation of the inner protoplasmic surface *Y* from the outer *X*. As the process continues the space, *S*, between them increases. *V*, vacuole. Optical section. Diagrammatic.

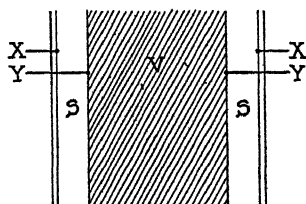


FIG. 2. A later stage of the process indicated in Fig. 1. Diagrammatic.

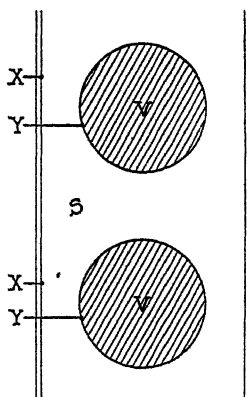


FIG. 3. A later stage of the process indicated in Fig. 2. The central vacuole has broken up into smaller vacuoles. Diagrammatic.

The sea water was employed because it is a balanced solution. The molar proportions of salts were⁵ approximately as follows: NaCl 1000, MgCl₂ 78, MgSO₄ 38, KCl 22, CaCl₂ 22. It had a Cl content of 0.52 m.

⁵ Cf. Osterhout, W. J. V., *Bot. Rev.*, 1936, 2, 283.

It is probable that the principal salt which penetrates is NaCl which in pure solution produces effects like those of sea water but at a lower osmotic pressure. This indicates that the penetration of NaCl is inhibited to some extent⁶ by CaCl₂.

These effects are reversible (with no disarrangement of the chloroplasts) if the cells are returned to tap water before the process has gone too far.

These relations may be strikingly demonstrated by staining⁷ the vacuole with brilliant cresyl blue or with neutral red but it is possible that these substances may have some direct effect.⁸

Similar and very striking results were obtained with *Chara Braunii*, Gmelin, whose cells are well adapted to such studies. This applies to a considerable extent to *Hydrodictyon reticulatum* (L.) Lagerh.

Very small cells of *Valonia macrophysa*, Kütz were kindly furnished by Dr. L. R. Blinks. These were nearly spherical and less than 2 mm. in diameter. When they were removed from Bermuda sea water (with a Cl content of about 0.58 M) and placed in sea water 1 part plus 3 M KSCN about 1 part, small local concavities appeared due to the simultaneous withdrawal of X and Y, without increasing the distance between the two layers.⁹ In some places, however, there was a small separation of the two layers. Very careful focussing is required to make sure that such separation has occurred.

The process is reversible if not allowed to go too far.

It would therefore seem that the inner and outer surfaces in *Valonia* differ. This conclusion is in harmony with the results of electrical experiments for when we form the chain¹⁰

Sap | Protoplasm | Sap

we obtain about 65 mv.¹¹

We may therefore conclude that in all these large cells the two non-aqueous protoplasmic surfaces differ in character.

Experiments somewhat similar in nature have been made on small plant cells by various investigators. In most cases these experiments involved very long

⁶ Cf. Osterhout, W. J. V., *Science*, 1911, **34**, 187. In the present experiments with NaCl the salt furnished by Merck and Co. "for biological use" was employed.

⁷ A few minutes in 0.25 per cent of brilliant cresyl blue or 0.05 per cent of neutral red at pH 8.5 is sufficient.

⁸ Strugger, S., *Ber. deutsch. bot. Ges.*, 1931, **49**, 453. Drawert, H., *Ber. deutsch. bot. Ges.*, 1938, **56**, 123. Küster, E., *Ber. deutsch. bot. Ges.*, 1940, **58**, 413.

⁹ In the normal state the distance between them is less than 10 microns.

¹⁰ See page 139.

¹¹ Damon, E. B., *J. Gen. Physiol.*, 1931-32, **15**, 525. The electrode in contact with X is negative in the external circuit to the electrode in contact with Y (as in *Nitella*).

exposure to stains which may in itself influence the result,¹² and the effects appeared very slowly and were often not reversible. Balanced solutions were not employed. In no case were electrical measurements made.

The fact that one part of the cell can be made to take water from another is significant and deserves further study.

SUMMARY

In *Nitella*, *Chara*, *Hydrodictyon*, and *Valonia* the inner and outer non-aqueous protoplasmic surface layers can be separated by certain plasmolytic agents which penetrate the outer surface more rapidly than the inner and hence raise the osmotic pressure of the protoplasm lying between them and cause it to increase in thickness by taking up water from the central vacuole.

We may therefore conclude that the two surfaces differ. This idea is confirmed by earlier electrical measurements which show that when sap is placed outside the cell the chain

Sap		Protoplasm		Sap
external				in vacuole

produces an E.M.F. of several millivolts.

¹² For the literature see Hartmair, V., *Protoplasma*, 1937, **28**, 582. See also footnote 8.

HOMEOSTATIC ADJUSTMENTS AFTER EXERCISE*

I. ACID-BASE EQUILIBRIUM OF THE BLOOD

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(Received for publication, August 20, 1943)

INTRODUCTION

Previous studies have shown that after severe exercise the pH and bicarbonate contents of the blood diminish (1, 3, 4, 7, 8, 15). The present report presents data on (a) the extent and nature of changes in acid-base balance of the blood which occur after work, and (b) the rate at which the acid-base balance returns to normal after maximal displacement. Simultaneous measurements of oxygen consumption, blood pressure, and pulse rate were made in order to assess the physiological changes induced by the exercise.

EXPERIMENTS

Subjects: Twelve male medical students, aged 18 to 24 years, served as subjects for the experiments. Duplicate experiments were performed on each subject with an interval of 1 week to 2 months between tests. Exercise used: Two forms of exercise were used in these experiments, *viz.* (a) leg exercise which consisted in climbing four flights of stairs (17.8 meters) at maximum speed, and (b) arm exercise performed on a bicycle ergometer arranged so that the subject turned the pedals by hand while lying supine (see Fig. 1).¹ The brake tension on the ergometer was adjusted for each subject so that the total work done against the instrumental friction was the same as the work done against gravity in the stair climb. The arm exercise was continued for 3 minutes.

Experimental Procedure

Each subject came to the laboratory at 7:30 a.m. in a fasting condition. After a 30 minute rest in the supine condition, the basal oxygen consumption was determined by the Tissot open circuit method (2). A Siebe-Gorman half-mask with mercury valves was used in collecting the expired air during three 8-minute periods. Carbon dioxide and oxygen determinations were made on samples of expired air using a 10 cc.

* Assistance in the preparation of these materials by the personnel of the Work Projects Administration O. P. Number 46503-631, Unit A8 and 65-1-08-62, Unit A8, is gratefully acknowledged. Thanks are due to Mr. T. Chernikoff, Miss K. H. Long, and Professor E. Ogden, who assisted in the work.

¹ Details of the construction and calibration of the bicycle ergometer used have been published previously (14).

mask, which had been securely attached to him before the running began, were connected to the recording spirometer (Fig. 1 and (14)).² A pneumatic cuff applied around the subject's ankle was inflated and a continuous recording of heart rate was made on a paper polygraph (14). Systolic and diastolic blood pressures were determined at 30 second intervals for the first 5 minutes, and at 1 minute intervals thereafter throughout the recovery period.³ Samples of finger blood were withdrawn before the exercise began and at 1, 2, 5, 10, 15, 30, 45, 60, and 90 minutes after the end of exercise for estimation of acid-base balance by the micro method (12). Continuous collection of expired air was made, and carbon dioxide and oxygen content was determined for each 10 liter sample in the Haldane apparatus. Duplicate experiments were made on each of the twelve subjects.

Treatment of Data

Oxygen Consumption.—From the expired air volumes and the $\text{CO}_2\text{-O}_2$ analyses, respiratory volume, oxygen consumption, and carbon dioxide eliminations were computed for each period. In preparing charts, of which Fig. 2 is an example, all metabolism results were plotted with abscissae at the middle of the time interval over which the sample of expired air was collected. An initial attempt at expressing the rate of recovery of oxygen consumption can be made by determining the parameter b in the empirical fit, $y = at^b + c$, where y is the oxygen consumption in cubic centimeters per minute per kilo at time t , c is the basal oxygen consumption, and a and b are constants.⁴ This method has been

² In the stair-climbing experiments it was found inexpedient to collect the small amount of air expired during the 15 to 20 seconds of exercise. When arm exercise was used, the subject breathed through the mask into one spirometer during the 3 minutes of exercise, but collection of expired air was begun in the other spirometer at the end of the exercise period.

³ The exact time at which the systolic and diastolic pressures were read was marked electrically on the paper polygraph. See (14) for a complete description of the apparatus.

⁴ That b measures this recovery rate at least for the range of values for which $t^4 \gg b^2$ (i.e., the only range for which this fit pretends to apply) follows from certain elementary considerations: Letting $y - c = z$, we can easily show that

$$\frac{1}{z} \frac{dz}{dt} = \frac{b}{t} \quad \text{so that} \quad b = t \left(\frac{1}{z} \frac{dz}{dt} \right) = tu$$

i.e., if t is plotted against $\frac{1}{z} \frac{dz}{dt} \equiv u$ the graph is a rectangular hyperbola.

The curvature of this hyperbola is

$$k = \frac{2bt^3}{(t^4 + b^2)^{3/2}}$$

whence if $t^4 \gg b^2$, it follows that the curvature is proportional to b .

previously used (Jenss and Shock (6)). Table I shows the values for b obtained in all experiments of this series (column I, first experiment, column II, second experiment).

Cardiovascular Data.—Pulse rate was counted over 10 second intervals from the polygraph record, and plotted as rate per minute shown in Fig. 2 with abscissae at the midpoint of the 10 second interval. Systolic and diastolic

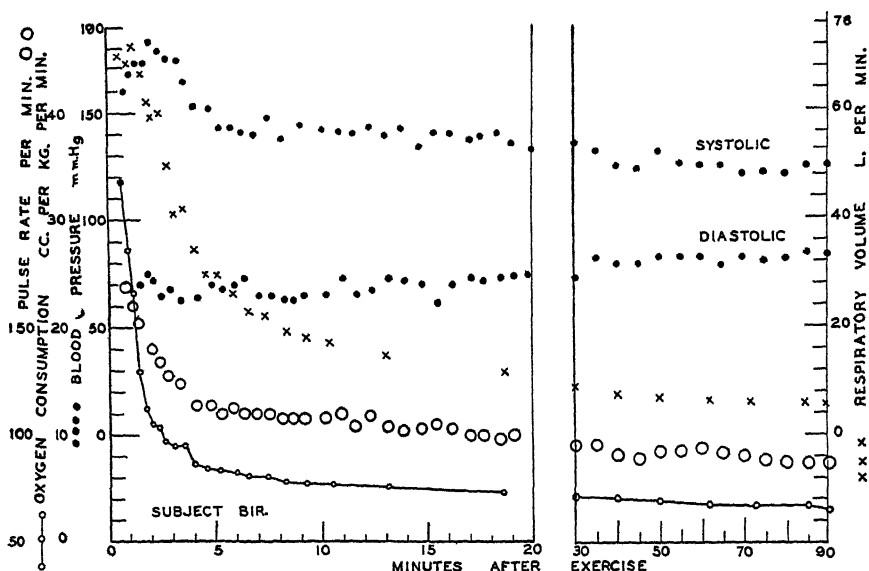


FIG. 2. Recovery of displacements of physiological functions produced by stair climbing in subject Bir. ●●● systolic and diastolic blood pressure, millimeters Hg.; xxx respiratory volume, liters per minute; ooo pulse rate, beats per minute; o-o-o oxygen consumption, cubic centimeters per kilo per minute. Exercise: 17.8 meters in 44.2 seconds. Basal data: height, 168.7 cm.; weight, 76.7 kilos; blood pressure, 122/73 mm.; respiratory volume, 6.46 liters per minute; pulse rate, 74 per minute; oxygen consumption, 3.25 cc. per kilo per minute (total 248 cc. per minute).

blood pressures were also plotted with abscissae corresponding to the actual time at which the measurement was made, as shown by the mark on the polygraph record.

Blood Analyses.—Cell volume, (V_c), pH, and total CO_2 content were determined for each blood sample by the micro method of Shock and Hastings (12). Bicarbonate content of the serum (BHCO_3), and carbon dioxide tension were calculated using a nomogram (5). Table II shows the blood data obtained in a sample experiment. (Columns 5, 6, 7, and 8 are calculated values.)

TABLE I
Coefficients of Recovery after Exercise

Subject	<i>b</i>		<i>k</i>		<i>t_m</i>		<i>C_m</i>	
	I	II	I	II	I	II	I	II
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Stair climbing								
Bir.....	1.008	1.078	0.94	0.74	10.0	9.0	17.2	15.1
Cast.....	1.118	1.021	1.29	1.30	5.0	4.5	9.5	13.7
Char.....	1.025	1.058	1.30	1.08	6.0	7.0	11.6	12.0
Gib.....	1.296	1.304	0.80	0.97	4.5	4.5	11.3	12.1
Ken.....	1.178	1.231	0.61	0.65	4.0	4.0	12.9	10.5
Fus.....	0.999	1.136	0.85	0.93	6.0	—	13.6	—
Arm exercise								
Cal.....	1.028	0.955	0.74	0.70	4.0	4.25	11.6	11.0
Dal.....	1.069	0.925	0.76	0.38	10.0	—	8.5	—
Fish.....	0.954	1.390	1.18	1.10	7.0	7.0	8.4	10.9
Fitz.....	1.182	—	1.63	0.99	2.0	—	4.7	—
Port.....	0.852	0.822	0.63	0.63	7.0	7.5	15.1	14.1
Rop.....	0.737	0.752	1.05	0.75	5.5	4.75	9.2	12.7

TABLE II
Displacement and Recovery of Acid-Base Balance of the Blood after Severe Exercise
Stair climbing; 17.8 meters in 44.2 seconds. Subject Bir.; weight 76.7 kilos.

Time after exercise	<i>V_e</i>	pH _s at 38° C.	[CO ₂] _b	[BHCO ₃] _b	[BHCO ₃] _s	pCO ₂	Δ[BHCO ₃] _b corrected
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
<i>min.</i>			<i>mM/l.</i>	<i>mM/l.</i>	<i>mM/l.</i>	<i>mm. Hg</i>	<i>mM/l.</i>
0.0	0.51	7.44	21.55	20.41	26.7	40.9	—
0.5	0.56	7.30	17.38	16.16	20.6	43.7	7.75
1.5	0.57	7.26	13.40	12.39	15.9	36.5	12.52
4.0	0.60	7.21	10.69	9.78	12.6	32.8	16.38
7.0	0.56	7.19	10.21	9.34	11.8	31.4	17.07
10.0	0.56	7.19	10.22	9.34	11.8	31.6	17.32
15.0	0.54	7.23	10.55	9.71	12.3	30.0	15.95
30.0	0.53	7.29	13.91	12.93	16.3	34.9	11.23
45.0	0.50	7.33	17.04	15.95	20.0	38.9	7.21
60.0	0.51	7.35	19.15	17.96	22.6	42.7	4.70
90.0	0.52	7.37	20.37	19.15	24.3	43.6	3.01

RESULTS

Acid Base Balance of the Blood.—Average curves of displacement of acid-base balance of the blood: Since the blood samples in all experiments were drawn at the same time intervals, it was possible to calculate average values for all experiments using the same kind of exercise. Figs. 3 and 4 show the results of such calculations for stair climbing and arm exercise, respectively. In comparing the two figures it is evident that although the same number of kilogrammeters of work were done in the two cases,⁵ the greater power developed in stair climbing produced greater changes in the acid-base equilibrium than did the corresponding arm exercise. For example, the maximum decrease in $(\text{BHCO}_3)_s$ averaged 12.1 mM per liter after stair climbing, while the decrease was only 9.1 mM per liter after arm exercise. The curves show that after maximal exertion, pH_s and $(\text{BHCO}_3)_s$ decrease, while the percentage of red cells (V_c) increases. The maximum decrease in bicarbonate content and pH of the blood do not occur until 7 minutes after the end of the exercise, on the average. Individual differences in the time of maximum displacement are shown in columns 5 and 6 of Table I. It should be noted that by this time the respiratory volume and oxygen consumption are well on their way to recovery (see Fig. 2). For instance in the experiment illustrated, the oxygen consumption has fallen from 950 per cent above basal immediately after exercise to only 100 per cent above basal at 7 minutes after exercise, when maximum changes in $(\text{BHCO}_3)_s$ and pH_s are recorded. Previous observers (4, 7) have found a similar delay in the appearance of maximum displacement of pH. Whether this delayed appearance of fixed acid in the blood is due to delayed lactic acid formation (8) or to a relatively slow rate of diffusion of fixed acid from muscle to blood cannot be ascertained from the data now available (7, 8).

Since neither values of blood-water concentration nor values of total blood and plasma volumes were made, it is impossible to analyze the increase in cell volume (V_c) into the effect of increased numbers of cells in circulation from blood depots and the effect of shifts in water from plasma to tissues. The fact that the maximum V_c is not found until 4 to 7 minutes after cessation of exercise lends support to the view that shifts in water are important.

A decrease in pCO_2 (average, 6 mm.) of arterial blood shows that alveolar respiration is more than adequate for the removal of carbon dioxide. These results also lend support to the view that in severe (anoxic) exercise the increased respiration is determined more by peripheral stimulation of the carotid and aortic mechanism through lowered pO_2 than through an increase in pCO_2 of the circulating blood (11).

Acid-Base Paths of Displacement.—Paths of displacement and recovery of acid-base balance of the blood after representative stair-climbing experiments

⁵ *I.e.*, work against the *external* or conservative forces.

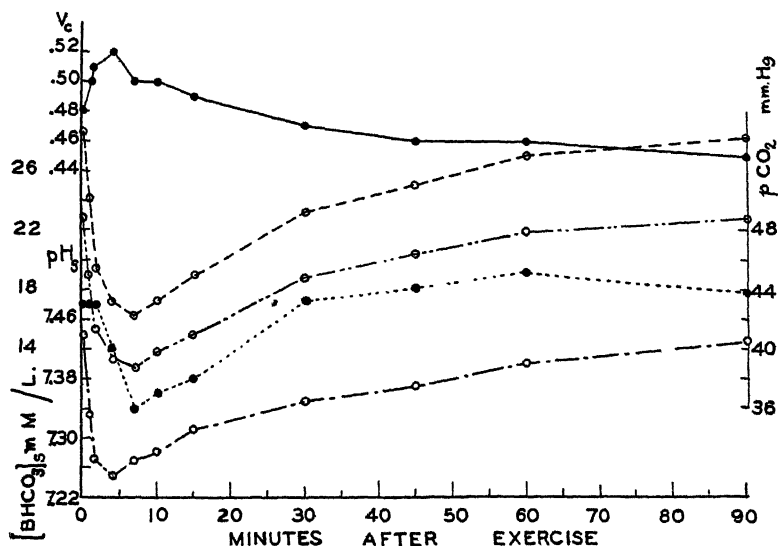


FIG. 3. Curves showing average displacement and recovery of acid-base equilibrium of the blood following stair climbing in six subjects. $\bullet \rightarrow V_c$; $\circ \text{---} \text{---} \text{---} \circ$ $[\text{BHCO}_3]_b$ mm per liter; $\circ \text{---} \text{---} \text{---} \circ$ $[\text{BHCO}_3]_i$ mm per liter; $\cdots \cdots \cdots$ $p\text{CO}_2$ mm. Hg.; $\text{---} \text{---} \text{---}$ pH, at 38°C . Points plotted at 0 time are measurements on blood drawn before the beginning of exercise.

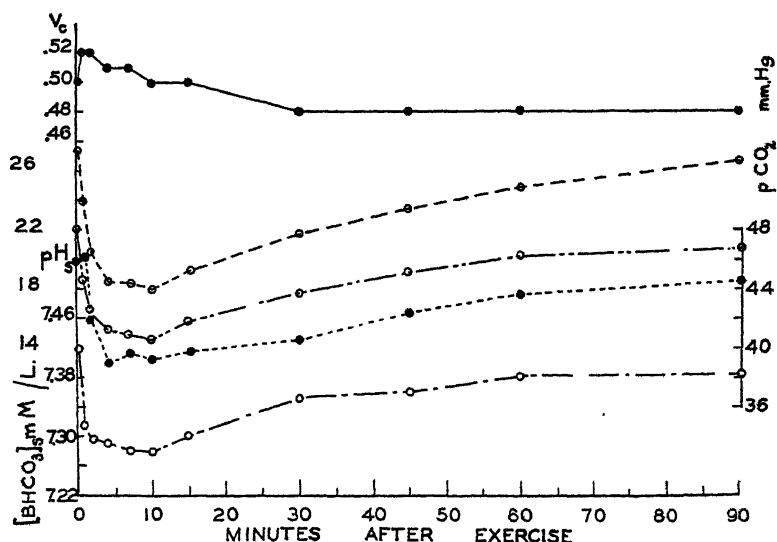


FIG. 4. Curves showing average displacement and recovery of acid-base equilibrium of the blood following arm exercise in six subjects. $\bullet \rightarrow V_c$; $\circ \text{---} \text{---} \text{---} \circ$ $[\text{BHCO}_3]_b$ mm per liter; $\circ \text{---} \text{---} \text{---} \circ$ $[\text{BHCO}_3]_i$ mm per liter; $\cdots \cdots \cdots$ $p\text{CO}_2$ mm. Hg.; $\text{---} \text{---} \text{---}$ pH, at 38°C . Points plotted at 0 time are measurements on blood drawn before the beginning of exercise.

are shown in Fig. 5. Similar paths after arm exercise are shown in Fig. 6. These charts were plotted on triaxial coordinate paper as described by Shock and Hastings (13).⁶ Duplicate experiments for each subject are placed one above the other in each figure. Inspection of these charts shows that the path of acid-base displacement after exercise follows the same general direction as that found previously after the ingestion of acidifying agents such as ammonium chloride (13). However, striking differences in the rate and extent of displacement are found when the present exercise experiments are compared with the results of oral administration of ammonium chloride (13). Much greater metabolic acidosis was produced by exercise than could be induced by the oral administration of ammonium chloride. Furthermore, recovery was much more rapid after exercise than after ammonium chloride ingestion.⁷ The path of recovery in all experiments was indicative of a period of "compensation," during which the pH tended to return to normal at a more rapid rate than did the bicarbonate content. This effect was produced in the organism by decreasing the $p\text{CO}_2$ with an increase in respiration.

Rate of Recovery of Bicarbonate Content of the Blood after Exercise.—In order to arrive at a quantitative estimate of the rate at which the bicarbonate content of the blood returned to normal after exercise, the data for each experiment were used to determine the "constant of elimination" as described in a previous report (13). Since with decreasing pH less base is bound by the proteins of the blood, the decrease in bicarbonate observed is less than the actual addition of fixed acid (15). By utilizing the buffer value of whole blood and correcting all observed values of $(\text{BHCO}_3)_b$ to pH 7.40, the true increase in fixed acid in the blood could be calculated. In making these calculations it was assumed that dB/dpH is 25 (10), where dB/dpH represents the change in millimoles of base, per liter of blood bound by blood buffers per unit change in pH between the pH limits of 7.2 and 7.6.⁸ From the calculated values of $(\text{BHCO}_3)_b$ at pH 7.4, the changes in $(\text{BHCO}_3)_b$ were calculated ($\Delta[\text{BHCO}_3]_b$ of column 8, Table II). From the plot of corrected changes in bicarbonate *vs.* time, we may obtain the time at which the maximum displacement of the acid-base balance was found (t_m), and the maximum decrease in bicarbonate, (C_m), which was observed at that time.

⁶ Constant pH lines run vertically (north and south); constant (BHCO_3) lines run northwest and southeast; constant $p\text{CO}_2$ lines run northeast and southwest. BHCO_3 and $p\text{CO}_2$ scales are logarithmic, while the pH scale is arithmetic. The small interior hexagon represents the limits of normal variation in males (13).

⁷ It should be noted that in the exercise charts, the time between measurements is indicated in minutes by the small figures. In the previous experiments (13) the time interval between points was $\frac{1}{2}$ to 1 hour.

⁸ Correction for changes in V_e was included in the formula for the value corrected to pH 7.4, $V_e 0.40$, of

$$\text{BHCO}_3 = \text{pH} (476 V_e + 8.2) \quad (10).$$

ACID-BASE PATHS

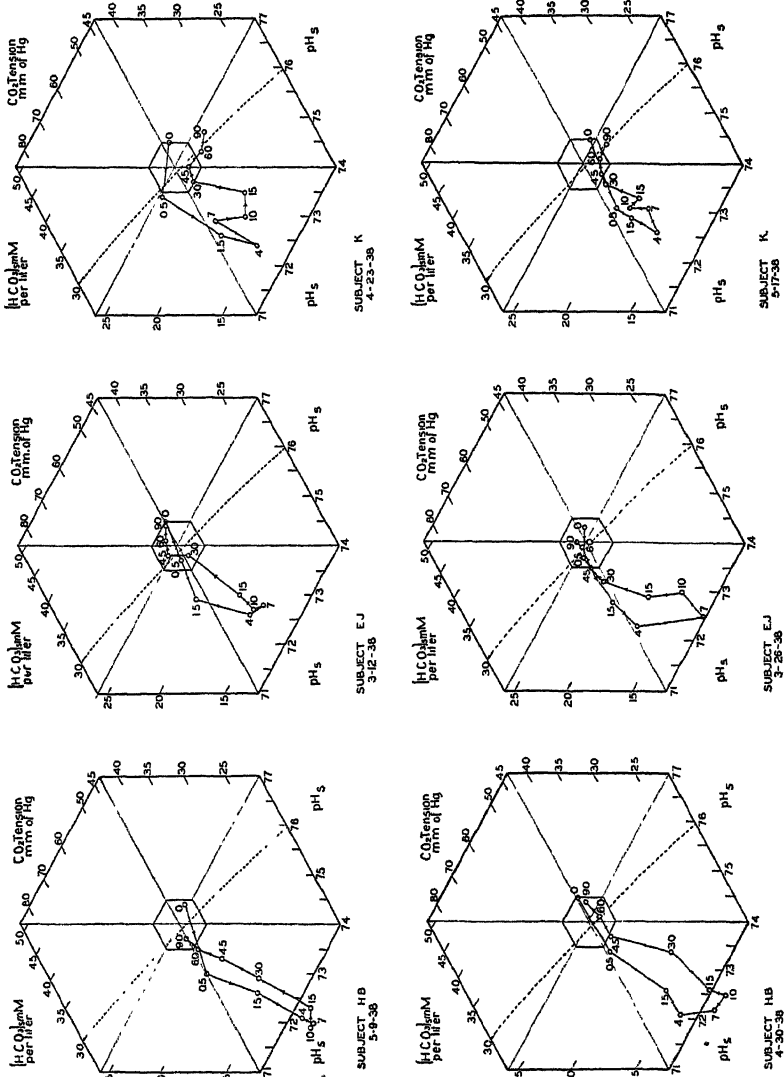


Fig. 5. Paths of displacement and recovery of acid-base equilibrium of the blood following stair climbing in three subjects. The numbers indicate minutes after exercise was completed.

ACID-BASE PATHS

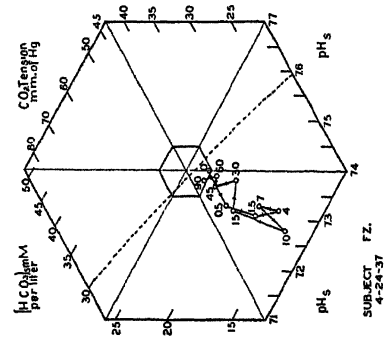
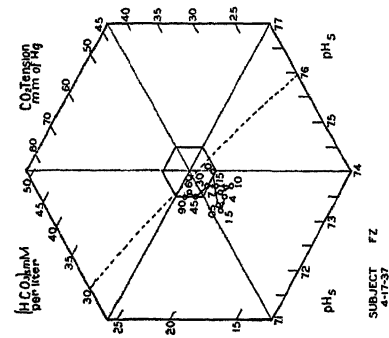
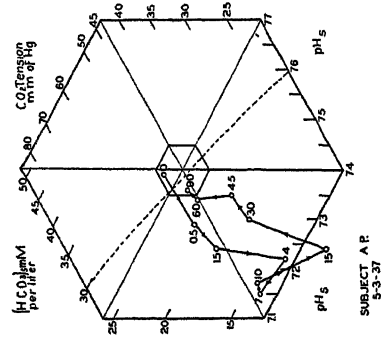
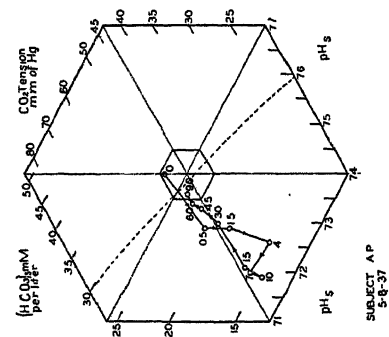
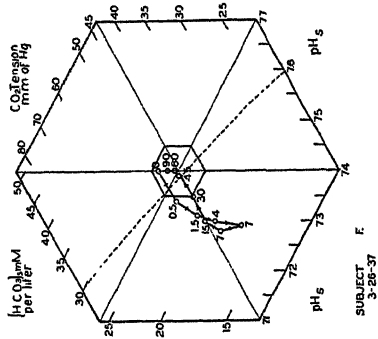
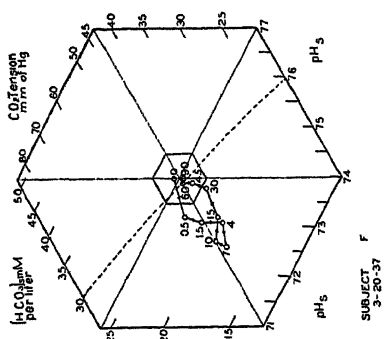


FIG. 6. Paths of displacement and recovery of acid-base equilibrium of the blood following arm exercise in three subjects. The numbers indicate minutes after end of exercise.

These curves also furnish the data for an empirical analysis presented previously (13), *i.e.* the fit of the equation, $c = ae^{kt}$, to the descending portion of the curve. In this equation c is the change in bicarbonate in mm/liter, t is the time after the end of exercise in hours, k is the so called "elimination constant," and a the initial value of c when the fit is assumed to hold for the entire range; k was determined from the slope of a free hand line fitted to a plot of $\log (\text{BHCO}_3)_t$ (at pH 7.4) against time in hours. An example is shown in Fig. 7.

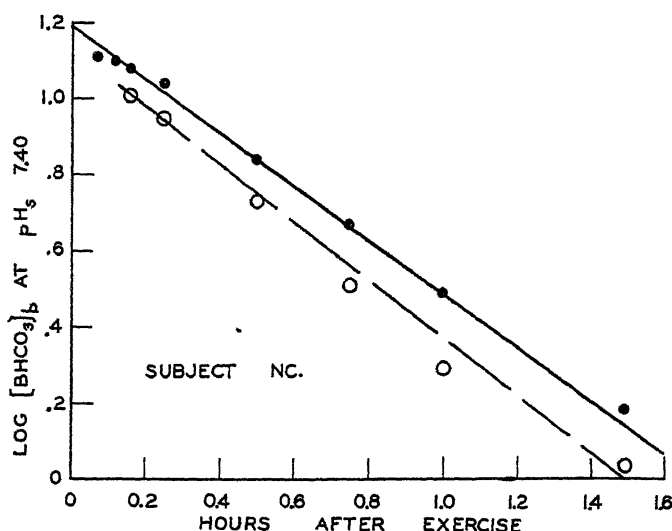


FIG. 7. Determination of elimination constant k for duplicate experiments (arm exercise) on subject NC. ○○○ Experiment 1, $k = 0.70$; ●●● Experiment 2, $k = 0.74$.

It might here be remarked that the foregoing fit was for purely representative purposes, and was not intended to be a theoretical interpretation of the process. We shall show later, however, that this equation, previously regarded as an empirical fit, is in fact an approximation to a rigorously derived theoretical equation (9).

Values of k calculated in the manner shown for all experiments are listed in Table I (columns 3 and 4) along with values for the oxygen recovery coefficient (b), the t_m ,⁹ and the C_m . Results show values of k ranging from -0.3 to -0.2 . Elimination constants previously reported for the oral administration of sodium bicarbonate and ammonium chloride ranged between -0.05 and -0.11 . Thus it is shown that fixed acids liberated into the blood stream after exercise are

⁹ t_m = time of maximum displacement of $(\text{BHCO}_3)_t$.

C_m = maximum decrease in $(\text{BHCO}_3)_t$ in mm per liter.

removed from the blood at a rate 10 to 20 times greater than the HCl produced from the oral administration of ammonium chloride. The greater rate of removal of acid produced by exercise may be interpreted as a reflection of the metabolism of lactate which may occur in other parts of the body—a removal mechanism which is not available for excess HCl.

SUMMARY

The rate at which displacement and recovery of the acid-base equilibrium of the blood occur in young adult males subjected to short periods of maximal exertion has been determined.

Displacement of acid-base equilibrium produced by severe exercise is along the fixed acid path, similar to the path of displacement produced by ingestion of acidifying agents such as ammonium chloride.

Maximum displacement of the acid-base equilibrium is not reached until 7 to 10 minutes after the cessation of exercise. By this time over 50 per cent of the displacement in oxygen consumption, respiratory volume, and blood pressure have disappeared.

A much greater metabolic acidosis was produced by exercise than could be induced by the oral administration of ammonium chloride.

Recovery from the metabolic acidosis produced by exercise was much more rapid (10 times) than was recovery from the acidosis produced by ammonium chloride.

After exercise the pH, returned to normal values more rapidly than did the bicarbonate content of the serum.

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A PHYSICAL THEORY OF ACID ANION DISPLACEMENT AND RECOVERY FOLLOWING EXERCISE

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When a muscle is stimulated to contraction some anion is produced which displaces bicarbonate ion from the blood. In the following note a simple theoretical model is presented whose behavior duplicates the variation of this ion concentration following exercise. Whether the ion is assumed to be lactate or pyruvate is immaterial for the analysis, since it is assumed that any increase in this anion is faithfully reflected by the attendant and measurable decrease in blood bicarbonate¹ according to the fast reaction, $\text{CH}_3\text{CHOH}\cdot\text{COOH} + \text{HCO}_3^- = \text{H}_2\text{O} + \text{CO}_2 + \text{CH}_3\cdot\text{CHOH}\text{COO}^-$. Our model then predicts the lactate (or pyruvate) ion concentration in muscle, blood, and removal fluid as a function of time. These predictions can be successfully tested in a variety of independent ways as will be shown.

The Model

Let us assume three unit-volume chambers separated from each other by permeable membranes, and filled with a solution of substance, S . We shall denote concentrations of S in chambers 1, 2, and 3, by c_1 , c_2 , and c_3 respectively. The condition of equilibrium of this system is obviously $c_1^0 = c_2^0 = c_3^0$. Suppose now that an excess of S is placed in chamber 1. The ensuing displacements would be governed by the rate equations for the process,²

$$(S \text{ in chamber 1}) \xrightleftharpoons[c_1(t)]{h_{12}} (S \text{ in chamber 2}) \xrightleftharpoons[c_2(t)]{h_{23}} (S \text{ in chamber 3})$$

where the h 's are in this case permeabilities of the separator membranes. Let us now postulate, however, that there exists some mechanical device on the membranes—a device to which energy is supplied from the exterior—preventing the back flow from chamber 2 to chamber 1, and from 3 to 2. This assumption is *mathematically* equivalent to setting $h_{21} = h_{32} = 0$, although it is realized *physically* that the permeabilities are the same in both directions through a membrane.

¹ Experimentally determined values of blood bicarbonate must be corrected to constant pH because of the changes in base bound by proteins at different pH.

² It is assumed that S is by some means mixed rapidly and evenly throughout the chambers after it enters them.

Next we introduce the new variables:

$$x_i = c_i - c_i^0$$

The new initial conditions in terms of these variables are $x_1^0 = c_1^0$, $x_2^0 = 0$, $x_3^0 = 0$. It will be seen that our system is now described by the equation,

$$\begin{array}{ccccc} (S \text{ in chamber 1}) & \xrightarrow{h_{12}} & (S \text{ in chamber 2}) & \xrightarrow{h_{23}} & (S \text{ in chamber 3}) \\ x_1(t) & & x_2(t) & & x_3(t) \end{array}$$

a mathematical form which is not a newcomer to biology when in chemical dress.

The foregoing is the model which is to simulate the 3 "chambers:" muscle cell, blood, removal cell (certain evidence suggests that the last is a kidney cell, although the possibility of liver cells is not excluded), and the diffusing substance, lactate ion.³ In order to make the application plausible we must explain: (1) The appearance of an excess amount of lactate, x_1^0 , in the muscle cell; (2) the irreversibility of passage of lactate from muscle to blood and from blood to removal cell. To do so we assume that: (1) Lactate ion is produced in pulse-like fashion within the muscle cell; *i.e.*, the reactions which give rise to the ion are much faster than its outward passage through the muscle cell membrane. (2) That the circulation of the blood reduces the lactate solute pressure directed backward to the muscle cell practically to zero, so that $h_{21}x_1 = 0$. But if the product of two numbers = 0 (with no further conditions), we are mathematically justified in setting either = 0; so we choose to set $h_{21} = 0$. This greatly simplifies the analysis and achieves the same physical effect as setting x_2 (neighborhood of membrane) = 0. By similar reasoning we set $h_{32} = 0$. In this case $h_{32}x_3 = 0$ (neighborhood of membrane) because again lactate is rapidly removed by some device (*e.g.* convection) other than diffusion. In the kidney this would be the passive formation of urine (meaning by "passive," in the absence of surface electric effects maintained by working cells), while in the liver it might be a rapid (as compared with diffusion) polymerization of lactate into glycogen.

It should perhaps be emphasized that despite the above assumptions, the h 's remain equal to the permeabilities of the two membranes in question; *i.e.*, that separating the interior of a muscle cell and the blood,⁴ and that separating the blood and the interior of the removal cell.

³ For the sake of brevity the term "lactate" or "lactate ion" is used in the subsequent discussion to indicate all anions produced which reduce the bicarbonate concentration in the blood.

⁴ Anatomically, of course, the muscle cell is bathed by extracellular fluid and is not in direct contact with blood, but since the ionic concentration of extracellular fluid is the same as that of blood plasma, we may regard the muscle cell as if it were in contact with the blood plasma.

THE EXPERIMENTS

Data for this analysis were obtained by the experimental procedures explained in detail in a previous paper (5). Briefly, samples of finger blood were drawn at 1 to 10 minute intervals following severe exercise in young male adults. Total CO_2 content, V_e , and pH_s were determined in each sample according to the micro method of Shock and Hastings (6). $(\text{BHCO}_3)_s$ and p_{CO_2} of the arterial blood were calculated (1). Furthermore, the change in bicarbonate at a pH_s of 7.4 and a V_e of 0.45 was computed for each sample (5). Corrected bicarbonate changes were used in the subsequent analysis.

With these considerations in mind we now turn to the test of our model. Once we identify $x_2(t)$ with the excess concentration of acid anion (lactate) in the blood, the two problems involved in the test become clear: we must find out how the *model* will behave, and we must devise some way of comparing the behavior of our model with the experiments. The first is an elementary problem in mathematics, and consists in finding the $x_i(t)$. The second will be a problem in the evaluation of constants.

Behavior of the Model

The system, $S(1) \rightarrow S(2) \rightarrow S(3)$, is a special case of the general reaction network $N_{i=1,2,\dots,m-1}$ of m substances. The mathematics of such networks will be presented in a paper now in preparation by one of us. Suffice to assert here that for $m = 3$, the constants, C_i^j in the solution,

$$x_i = \sum_{j=1}^{i-m-1} C_i^j e^{-k_j t} + C_i^m$$

are the following:

$$\left. \begin{aligned} C_3^1 &= -\frac{k_2 x_1^0}{k_2 - k_1} \\ C_3^2 &= -\left(x_2^0 - \frac{k_1}{k_2 - k_1} x_1^0\right) \\ C_3^3 &= x_1^0 + x_2^0 + x_3^0 \\ C_2^1 &= \frac{k_1}{k_2 - k_1} x_1^0 \\ C_2^2 &= x_2^0 - \frac{k_1}{k_2 - k_1} x_1^0 \\ C_1^1 &= x_1^0 \end{aligned} \right\} \quad (1)$$

Substituting these constants into the solution, restricting our problem to the case where $x_1^0 \neq 0$, $x_2 = x_3 = 0$, and interpreting the velocity constants k as permeabilities h , we obtain the well known relations:

$$x_1 = x_1^0 e^{-h_1 t} \quad (2)$$

$$x_2 = \frac{h_1 x_1^0}{h_2 - h_1} (e^{-h_1 t} - e^{-h_2 t}) \quad (3)$$

$$x_3 = \frac{x_1^0}{h_2 - h_1} (h_1 e^{-h_2 t} - h_2 e^{-h_1 t}) \quad (4)$$

From equations 2, 3, and 4 it is possible to plot the graphs of the concentrations.

The Graphs of the Concentrations.—(a) The x_1 : We have from 2, $x_1 = x_1^0 e^{-h_1 t}$. When $t = 0$, $x_1 = x_1^0$. When $t = \infty$, $x_1 = 0$. x_1^0 is zero only for $t = \infty$. This suffices to get an idea of the nature of the function; it is the familiar exponential decay curve, with x_1^0 as its intercept on the x_1 axis. (b) The x_2 : Evidently the quantity in parentheses in equation 3 is zero for $t = 0$ and for $t = \infty$, but for no other positive t .

$$\frac{dx_2}{dt} = \frac{h_1 x_1^0}{h_2 - h_1} (h_2 e^{-h_2 t} - h_1 e^{-h_1 t})$$

is zero when

$$\frac{h_2}{h_1} e^{(h_2 - h_1)t}, \text{ i.e., } t = \frac{1}{h_2 - h_1} \log \frac{h_2}{h_1} \quad (5)$$

This equation has only one root, so we know that there is but one maximum or minimum between 0 and ∞ . Now when $t = 0$, x_2^1 reduces to $h_1 x_0$. Since $h_1 \geq 0$ from physical considerations, we know that the curve passes through the origin with a positive slope. Furthermore, it will again return to the t -axis at ∞ , and it will not touch in between. Evidently, then, the single point at which its slope is zero for $0 < t < \infty$ must be a maximum.

$$\frac{d^2 x_2}{dt^2} = \frac{x_1^0 h_1}{h_2 - h_1} (h_1^2 e^{-h_1 t} - h_2^2 e^{-h_2 t}) = 0$$

when

$$t = \frac{2}{h_2 - h_1} \log \frac{h_1}{h_2} \quad (6)$$

If by t_m we denote the abscissa of the maximum, and by t_i that of the inflection point, then from equations 5 and 6 we find the relation that $t_i = 2t_m$. We now have enough information to sketch the graph (x_2 , in Fig. 1). (c) The x_3 :

From equation 4, when $t = 0$, $x_3 = 0$, and when $t = \infty$, $x_3 = x_1^0$. Differentiating,

$$\frac{dx_3}{dt} = x_1^0 \left[\frac{h_1 h_2}{h_2 - h_1} (e^{-h_1 t} - e^{-h_2 t}) \right] \quad (7)$$

This function is zero for $e^{-h_1 t} = e^{-h_2 t}$, that is, for $t = 0$, and again for $t = \infty$.

$$\frac{d^2 x_3}{dt^2} = \frac{h_1 h_2 x_1^0}{h_2 - h_1} (h_2 e^{-h_2 t} - h_1 e^{-h_1 t}) \quad (8)$$

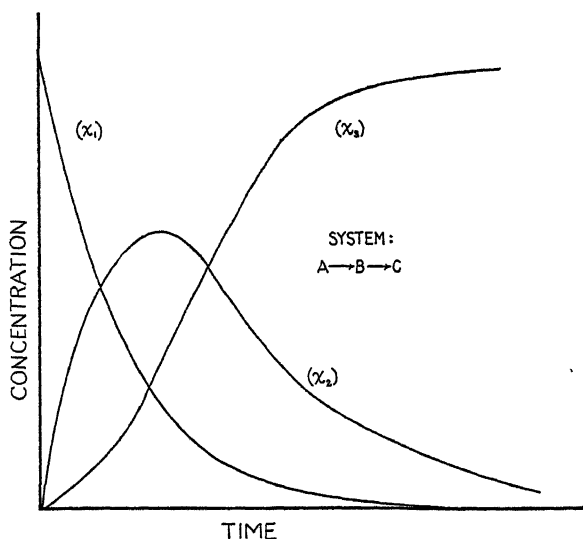


FIG. 1. Graph showing theoretical time-concentration curves for model derived from rational equations. x_1 = concentration of S in first cell (muscle); x_2 concentration of S in intermediate cell (blood plasma); x_3 = concentration of S in third cell of system (removal cell).

Comparing with equation 5, we see that x_3 has an inflection simultaneous with the maximum of x_2 .

Using the deductions of (a), (b), and (c), the graphs of the concentrations x_1 , x_2 , and x_3 were drawn. (See Fig. 1.) The striking resemblance between the curve for x_2 and the smoothed experimental curves (Figs. 2 and 3) is obvious.

Fitting of the Curve x_2

Equation 3 contains three constants which must be evaluated; consequently from the experimental graph we must be able to get three independent measures

yielding three independent equations in the constants. The time of the maximum and the maximum concentration (hereinafter referred to as t_m and C_m) are readily obtained. It might be thought at first glance that the abscissa of the inflection would furnish a suitable third point. This is not the case, inasmuch as it is quite impossible to read this measure from an experimental curve with an accuracy comparable to the abscissa of the maximum. Nevertheless it may serve as a rough check. When x_2 drops off very rapidly, a good approximation to the area under the curve x_2 can be obtained. This condition is well met by our data, so we decided to use the area under x_2 (hereinafter denoted by A), measured with an ordinary planimeter as our third experimental parameter.

Having decided upon the three experimentally imposed conditions, we must derive the corresponding equations in the constants h_1 , h_2 , and x_1^0 .

One of these has already appeared. From equation 5 we readily obtain our first equation

$$t_m = \frac{1}{h_2 - h_1} \log \frac{h_2}{h_1} \quad (9)$$

Furthermore, for $t = t_m$, $e^{-h_2 t_m} = \frac{h_1}{h_2} e^{-h_1 t_m}$, so that

$$\begin{aligned} C_m &= \frac{x_1^0 h_1}{h_2 - h_1} \left[e^{-h_1 t_m} - \frac{h_1}{h_2} e^{-h_1 t_m} \right] \\ &= \frac{h_1}{h_2} x_1^0 e^{-h_1 t_m} \end{aligned} \quad (10)$$

The area under the curve is readily found;

$$A = \int_0^\infty \frac{h_1 x_1^0}{h_2 - h_1} (e^{-h_1 t} - e^{-h_2 t}) dt = \frac{x_1^0}{h_2}$$

whence,

$$A h_2 = x_1^0 \quad (11)$$

Equations 9, 10, and 11 constitute our system to find the constants of the fit. There is one peculiarity about this system, however. In rearranging equation 5 it was necessary to multiply through by $(h_2 - h_1)$. This introduced a solution $h_2 = h_1$. The solution is not a valid one because we are using for x_2 the solution which assumed the h 's distinct.

Solution of the System.—Substituting from equation 11 into 10, we obtain a simple equation for h_1 ,

$$h_1 e^{-h_1 t_m} = \frac{C_m}{A} \quad (12)$$

The function $h_1 e^{-h_1 t_m}$ is zero for $h_1 = 0$ and $h_1 = \infty$ ($h_1 \geq 0$). Differentiating once we see that there is a maximum when

$$e^{-h_1 t_m} = h_1^2 e^{-h_1 t_m} \quad \text{or} \quad h_1 = 1$$

This fact indicates that if any solutions at all exist for h_1 , two will exist. Moreover, if we solve equation 9 for $\frac{h_1}{h_2}$ and substitute into equation 10, we find that

h_2 must satisfy the very same equation as h_1 , namely, $h_2 e^{-h_2 t_m} = \frac{C_m}{A}$. This

equation will also have two roots, if any, and they will be the same ones as for h_1 . Since we know $h_1 \neq h_2$, the only conclusion possible is that one of the solutions of $x = e^{-t_m x}$ is h_1 and the other is h_2 . Just which is which can be easily ascertained by solving the combination of equations 10 and 11, and choosing one of the possible combinations of h_1 and h_2 . If this combination satisfies equation 5, it is the correct combination; if not, then the other pair must be. The best way to solve equation 12 is to graph it in the interval 0

$< x < \infty$. The intercepts of the curve with the straight line $y = \frac{C_m}{A}$ give the approximate roots. By any one of the numerical methods, *e.g.* the Newton-Raphson, the true values can be found as closely as desired. The value of h_1 which makes $h_1 e^{-h_1 t_m}$ a maximum is $h_1 = \frac{1}{t_m}$, whence the maximum value of

$h_1 e^{-h_1 t_m}$ is $\frac{1}{t_m e}$. Clearly, in order for equation 12 to have a solution, it must be that

$$\frac{1}{t_m e} \geq \frac{C_m}{A} \quad \text{or} \quad \frac{A}{t_m C_m} \geq e \quad (13)$$

The equation-inequality 13 forms the best criterion as to whether or not equation 3 will fit given experimental data, for its left hand member contains only constants of the experiment, and the right hand member is a universal constant. It is also obvious that the condition of equation 13 is invariant to changes in units either of t or C , for both of its sides are dimensionless quantities. Experimental curves which to all appearances seem alike can be shown to involve fundamentally different processes under equation 5. For instance, the fatigue-recovery curves and the change in skin resistance curves following stimulation are identical to cursory inspection, but the former satisfy equation 5 while the latter do not.

The fitting of the curve may now be summarized as follows: (1) From the data of $[\text{HCO}_3^-]_s$ vs. time are obtained the data $\Delta[\text{HCO}_3^-]_s$ vs. time (normal is taken as $[\text{HCO}_3^-]_s$ for $t = 0$). These latter are accurately plotted on coordi-

nate paper and a smooth curve is drawn through them (2) From the curves thus obtained the following parameters are tabulated: t_m , C_m , and A . (3) An accurate graph of the function $y = xe^{-x}$ vs. x is plotted. The same graph serves for all computations. (4) The abscissas of the intersections of $y = xe^{-x}$ and the straight lines $y = \frac{t_m C_m}{A}$ then yield the desired values of h_1 and h_2 .

TABLE I

Subject	Date	t_m	C_m	A	h_1	h_2
		<i>min.</i>	<i>mg/l.</i>			
1 J. A.....	3-12-38	7.0	13.3	398.9	0.0475	0.319
2 J. A.....	3-26-38	7.0	13.9	377.6	0.0536	0.299
3 G. I.....	4- 2-38	4.5	11.3	348.5	0.0387	0.674
4 G. I.....	4- 9-38	4.5	12.1	280.7	0.0556	0.576
5 C. H.....	2-19-38	6.0	11.6	258.4	0.0673	0.334
6 C. H.....	3- 5-38	7.0	12.0	219.8	0.0560	0.291
7 F. I. S.....	4-17-37	2.0	4.7	156.0	0.0335	2.132
8 F. I. S.....	3-20-37	7.0	8.4	286.2	0.0381	0.357
9 F. I. S.....	3-26-37	7.0	10.9	381.5	0.0371	0.364
10 D. A.....	2-20-37	10.0	8.5	327.2	0.0379	0.208
11 K. E.....	5-17-38	4.0	10.45	381.8	0.0310	0.863
12 K. E.....	4-23-38	4.0	12.9	504.5	0.0285	0.886
13 R. O.....	3- 6-37	4.75	12.65	587.9	0.0240	0.746
14 R. O.....	2-27-37	5.5	9.2	265.8	0.0445	0.477
15 C. A.....	2-28-38	4.5	13.7	230.9	0.0880	0.450
16 C. A.....	2-14-38	5.0	9.5	234.8	0.0538	0.505
17 C. A. L.....	3-13-37	4.0	11.6	425.1	0.0305	0.864
18 C. A. L.....	3-15-37	4.25	11.0	509.0	0.0235	0.866
19 B. I.....	4-30-38	10.0	17.2	713.8	0.0338	0.223
20 B. I.....	5- 9-38	9.0	15.1	748.4	0.0255	0.300
21 F. U.....	3-19-38	6.0	13.6	413.4	0.0425	0.428
22 P. O.....	5- 3-37	7.0	15.1	771.6	0.0229	0.447
23 P. O.....	5- 8-37	7.5	14.1	736.4	0.0227	0.407

(5) x_1^0 is finally obtained as Ah_2 . (6) Theoretical points are now calculated by means of equation 5.

THE RESULTS

The foregoing analysis has been applied to 23 experiments. The calculations are summarized in Table I. Figs. 2 and 3 illustrate the agreement obtained between theoretical and observed changes in acid anion concentration with time. The line represents the smooth curve through the experimental points (crosses). The circles are theoretical points calculated by substituting values of h_1 and h_2 into equation 5.

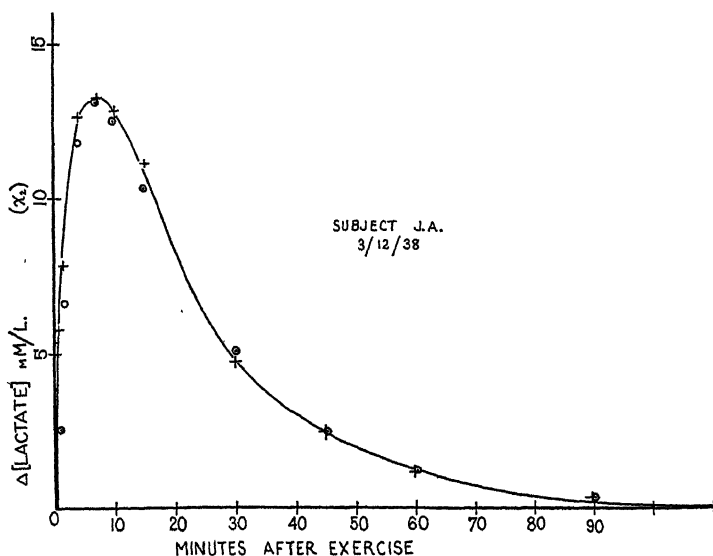


FIG. 2. Comparison of experimental and theoretical values of acid anion in blood following exercise. Experimental values shown as +; $t_m = 7.0$; $C_m = 13.9$; $A = 377.6$. Calculated values; $h_1 = 0.0536$; $h_2 = 0.299$. Curve shows free hand fit of experimental values. Theoretical values shown as O.

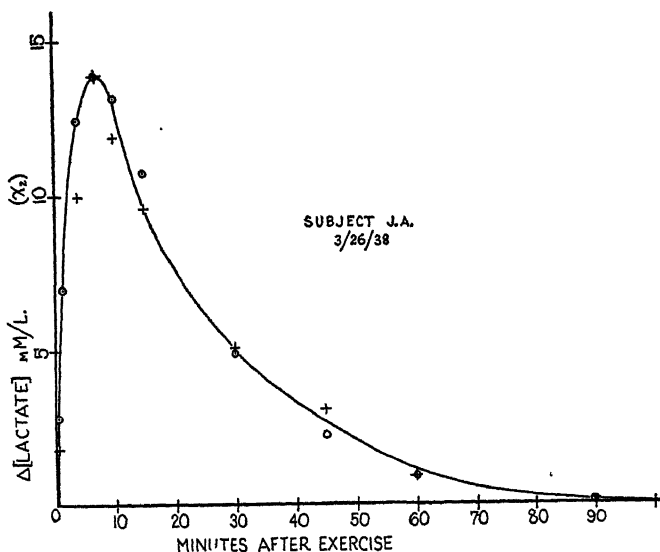


FIG. 3. Comparison of experimental and theoretical values of acid anion in blood following exercise. Experimental values shown as +; $t_m = 7.0$; $C_m = 13.3$; $A = 398.9$. Calculated values; $h_1 = 0.0475$; $h_2 = 0.319$. Theoretical values shown as O.

The permeabilities h_1 and h_2 average 0.0403 and 0.5845 respectively.⁵ For a particular subject they tend to remain the same in experiments performed on different days.

DISCUSSION

The comparison of model and reality as outlined in the Introduction is completed by the table and graphs given above. The model has, however, implications beyond the available data. These implications, if checked by experiments of an entirely different sort, lend important support to the theory, and thus are also worthy of consideration.

The theory predicts that the curve for x_3 , the lactate concentration in the removal cell or any phase connected with it, should have the general nature suggested by x_3 of Fig. 1. Experimental evidence which substantiates this point has been obtained by Johnson and Edwards (2). The curve for lactate ion concentration in the urine following exercise is of this sort. The inference, as already pointed out, is that the kidney cells are the removal cells required by the model. There is, of course, no way of measuring the lactate ion concentrations *inside* of muscle cells, but if one accepts the theory, these concentrations are necessarily given by $x_1(t)$.

The other implication has to do with the numerical magnitude of the permeabilities obtained. Expressed in millimoles and minutes, these have already been given as about 0.04 and 0.58. In view of the fact that the molecular weight of lactic acid is 90, the factor which converts the above h 's to permeabilities in centimeter-gram-second units is $0.090/60$. This gives 5.94×10^{-5} and 75×10^{-5} respectively. The "arbitrary permeability" is usually taken as 10^{-5} (4). Thus it is obvious that the h 's we have obtained are

⁵ Without the aberrant value of subject F. I. the average is 0.480. Quantitative description of recovery curves has been attempted by Shock and Hastings (7), and others (3). The first named investigators performed an empirical analysis based on the fitting of the curve,

$$x = Ce^{-kt}$$

It is readily seen that this curve is a special case of the one here proposed, for which $h_1 = h_2$. In this event we may set

$$\frac{Ah_1h_2}{h_2 - h_1} \cong -Ah_2 \quad \text{and} \quad e^{-h_1t} \cong 0,$$

so that

$$x_2 = Ah_2 e^{-h_2t}$$

The forms are identical if we identify h_2 with k , and C with Ah_2 . The values obtained by Shock and Hastings for k should therefore approximate the values here gotten for h_2 . This is roughly the case.

very reasonable values. It would be highly fortuitous that we should happen upon such magnitudes merely by chance, especially in view of all the previous correlations.

In summary it can be said that the phenomenon of acid displacement and recovery following exercise can be plausibly explained by our model, when reasonable (as shown by *independent* lines of reasoning) values of h are assumed. Imbedded in this solution, however, is the perhaps more important suggestion that h values can be obtained from the integral properties of systems, as here demonstrated. Thus these values of h were obtained under perfectly physiological, intact organism conditions, a feat which direct experimentation could not accomplish. The mathematical analysis of similar cases thus offers unlimited possibilities in the realm of intact animal research.

While it is logically wrong to claim uniqueness for a model, it is quite permissible to rule out certain other rival models. Among them would be a simple one purporting to give the *chemical* kinetics of lactate production. We have no doubt that the *chemical* kinetics will some day be elucidated, even though they may not be the governing reaction, but the necessary empirical information and the mathematical techniques for doing so are as yet unavailable. It may be that the lactate variations will be governed primarily by the rates of chemical reactions, but it can be said with great surety that no simple chemical models, such as have been suggested, could ever account for a reacting system of such complexity, involving catalysis at so many points.

SUMMARY

A simple theoretical model has been presented whose behavior duplicates the variation in bicarbonate ion concentration in the blood following exercise. Methods for the evaluation of the constants of rational equations to describe the concentration in muscle cells, in blood plasma, and in removal cells, of the anions produced in exercise have been devised. These methods have been applied to experimental data from 23 experiments, and a close agreement between the observed and theoretically predicted values for blood plasma has been found. From the mathematical analysis of the data values for permeability of acid anions produced in exercise have been estimated as 75×10^{-5} and 5.9×10^{-5} cm. per sec. between muscle cell and blood (extracellular fluid) and between blood plasma and removal cells respectively.

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THE PREPARATION AND PROPERTIES OF HIGHLY PURIFIED ASCORBIC ACID OXIDASE

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The initial observation that certain plant tissues catalyze the aerobic oxidation of vitamin C (*l*-ascorbic acid) was made by Szent-Györgyi in 1928 (1). The existence in cabbage leaves of a specific enzyme "hexoxidase"—called "ascorbic acid oxidase" by later workers—was postulated by Szent-Györgyi (2) in 1930. Since that time numerous investigators (3–10) have described the preparation and properties of ascorbic acid oxidase from a variety of plant sources. Some of these preparations have been relatively impure, and a comparison of the properties of ascorbic acid oxidase from different sources is difficult, since it is our experience that the properties vary markedly with the degree of purity of the enzyme. As nearly as can be judged, the preparation described by Lovett-Janison and Nelson (10) from the yellow summer crook-neck squash (*C. pepocondensa*) appears to be the most highly purified of those reported to date. These investigators clearly showed that the enzyme was a copper-protein with a proportionality existing between the copper content and the enzyme activity. Their best preparation contained 0.15 per cent copper and had an activity of 430 units per microgram (γ) of copper and 630 units per mg. of dry weight. The enzyme in dilute solutions was colorless, but in concentrated solutions (about 8 to 10 mg. enzyme protein per cc.) was green. Intermediate concentrations of the enzyme were pale blue to greenish blue in color.

At about the same time Stotz (8) described an enzyme preparation from cucumbers which had a copper content as high as 0.25 per cent, and an activity of 850 units per mg. dry weight. At first glance it would appear that this preparation was considerably more pure than that of Lovett-Janison and Nelson. However, the activity unit used by Stotz is smaller by a factor of six, and a comparison of the preparations on the same unit basis¹ shows the preparation

¹ The unit used by Stotz is based on an oxygen uptake of 100 mm.³ per hour during the enzymatic oxidation of ascorbic acid, while that of Lovett-Janison and Nelson is based on an oxygen uptake of 10 mm.³ per minute, or 600 mm.³ per hour. Converting the Stotz value of 850 units per mg. dry weight to Lovett-Janison and Nelson units gives a value of 141 units per mg. dry weight as compared to 630 units per mg. dry weight for the Lovett-Janison and Nelson preparation. Carrying the comparison still further, it can be seen that with the high value of copper reported by Stotz, the activity per γ of copper becomes very much lower than that reported by Lovett-Janison and Nelson; *i.e.*, 57 units per γ of copper as compared to 430 units per γ of copper.

of Lovett-Janison and Nelson to be over four times more active per milligram dry weight than the preparation described by Stotz. Furthermore, it would seem that the latter preparation either contained considerable non-enzyme copper, or the proportionality factor between enzyme activity and copper is very much lower for cucumber enzyme than for the squash enzyme.

The purpose of this communication is to describe the preparation from summer crook-neck squash of an ascorbic acid oxidase solution having approximately twice the activity per milligram dry weight as that of Lovett-Janison and Nelson; and having a copper content of 0.24 per cent. Secondly, some of the more interesting properties of this highly purified preparation are compared with those of previously described preparations of lower purity.

Purification of Enzyme from Summer Squash

The procedure of Lovett-Janison and Nelson (10) for the preparation of the oxidase was followed with a few changes. Since these changes resulted in a purer preparation with a higher yield, the modified procedure is described:—²

Step I. Crude Juice.—Twenty bushels of yellow summer squash were peeled; the rinds were minced to a fine pulp and the pulp subjected to hydraulic pressure to remove all the juice. At this point 100 liters of crude juice were obtained, having a total oxidase activity of 4,500,000 units and an activity of 1.2 units per mg. dry weight. (For the definition and determination of ascorbic acid oxidase activity see (10). The pH of the crude juice was 5.9. A small amount of sodium tetraborate was added to bring the pH to 7.6. The crude juice was clarified by adding molar barium acetate (10 cc. per liter of juice) and the resulting suspension was allowed to settle overnight before syphoning off the supernatant liquid. This operation removed considerable color and some extraneous protein, without causing any appreciable loss of oxidase activity.

*Step II. Fractionation by Ammonium Sulfate*³.—Barium ion was removed from the clarified juice by adding $(\text{NH}_4)_2\text{SO}_4$ to 0.3 saturation at room temperature (210 gm. per liter of juice). The precipitate was filtered off immediately. The enzyme was next precipitated from the filtrate by more $(\text{NH}_4)_2\text{SO}_4$ to 0.6 saturation and the precipitate was dissolved in about 9 liters of M/15 Na_2HPO_4 solution. This solution contained 4,390,000 units or 97 per cent of the enzyme as compared to 85 per cent of the enzyme at this point in the Lovett-Janison and Nelson procedure. Activity of the preparation was 88 units per mg. of dry weight.

² Chemicals used in the enzyme preparation were: Barium acetate, $\text{Ba}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$, Eimer and Amend, technical grade; ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$, General Chemical, granulated white grade; disodium phosphate, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, General Chemical, A.C.S. specifications; magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, General Chemical, technical grade; lead acetate, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$, Eimer and Amend, c. p. grade; and litharge, PbO , General Chemical, c. p. grade.

³ All filtrations except those involving the lead-acetone precipitates were filtered with suction through a $\frac{1}{4}$ to $\frac{1}{2}$ inch pad of celite (Johns-Manville No. 535). For the lead precipitates a $\frac{1}{2}$ inch pad of standard super-cel (Johns-Manville) was used.

Step III. Fractionation by Magnesium Sulfate.—The protein in the above solution was fractionated by the use of magnesium sulfate. The first fraction was precipitated by the addition of 500 gm. of magnesium sulfate per liter of solution, and contained enzyme along with inactive protein. However, in accordance with the experience of Lovett-Janison and Nelson, this precipitate could not be easily purified much further and was therefore discarded. More magnesium sulfate was added to the filtrate (about 400 gm. per liter) and the resulting precipitate contained the rest of the oxidase. The precipitate was filtered and dissolved in about $2\frac{1}{2}$ liters of $M/15$ Na_2HPO_4 solution. After dialyzing against running tap water for 2 days (temperature about $15^\circ C.$) the volume of the solution increased to about 3 liters. The solution contained 3,170,000 units or 70 per cent of the original activity and had an activity of 150 units per mg. dry weight.

Step IV. Fractionation by Alumina Adsorption.—The solution above was next treated with alumina reagent (11) (5 cc. of alumina per 100 cc. of solution) and filtered immediately. The alumina adsorbate, heavy with extraneous protein, was discarded although it also had considerable oxidase activity. The filtrate (now of a volume of 3150 cc. and containing 2,160,000 units) was treated with 0.1 saturated lead subacetate solution (1 cc. per 600 cc. of filtrate) to precipitate more extraneous protein before adsorbing the major portion of the enzyme to alumina. This was accomplished by adding alumina reagent (10 cc. per 100 cc. of solution) to the filtrate from the lead precipitation. The adsorbate was filtered off immediately, eluted with $M/15$ Na_2HPO_4 solution, and dialyzed overnight against running tap water (temperature about $15^\circ C.$). At this point the volume of the solution was 1500 cc., and contained 1,200,000 units or 27 per cent of the original activity. The solution had an activity of 232 units per mg. dry weight.

*Step V. Fractionation by Lead Subacetate*⁴.—To continue the purification, each 100 cc. of the above solution was treated with 10 cc. of dry-ice cooled acetone and then 1 cc. of 0.1 saturated lead subacetate solution. This first lead precipitation was heavy and contained considerable enzyme; however, it was discarded since experience had shown that the enzyme could not be easily separated from the extraneous protein in the precipitate. A second precipitate was obtained by treating the filtrate with 5 cc. more of the 0.1 saturated lead subacetate solution per 100 cc. of filtrate after adding cold acetone as before (10 cc. per 100 cc. of filtrate). The precipitate was filtered off and saved.⁵ The filtrate from this step was again treated with cold acetone as before and then with 15 cc. of the 0.1 saturated lead subacetate solution per 100 cc. of filtrate

⁴ The lead subacetate solution was prepared as follows: 420 gm. of $Pb(C_2H_3O_2)_2 \cdot 3H_2O$ and 140 gm. of PbO were ground in a mortar and mixed with 1400 cc. of water in a glass-stoppered bottle. After thorough shaking, it was allowed to stand for a week at room temperature and then filtered. One volume of the filtrate was diluted to ten volumes with water to make 0.1 saturated lead subacetate solution.

⁵ The second lead acetate precipitate was dissolved in $M/15$ Na_2HPO_4 solution, which upon dialysis for 2 days, yielded a blue-green precipitate. The precipitate was redissolved in 10 cc. of 0.1 M (Cu^{++} -free) Na_2HPO_4 solution to give a blue-green solution having an activity of 5750 units per cc. Calculations indicated this solution to have an activity of about 640 units per mg. dry weight of protein material. No measurements of its copper content were made.

and again filtered. The precipitate was small and, although it contained about 100,000 units of activity, it was discarded as attempts at further purification failed. Further treatment of the filtrate with acetone lead subacetate failed to produce precipitation.

Step VI. Adsorption to Alumina and Dialysis.—The filtrate from the lead acetate treatment was highly active (total activity, 350,000 units) and was treated with alumina reagent (5 cc. per 100 cc. of solution). This operation was performed immediately in order to remove the active enzyme from the solution; thus preventing its inactivation by the Pb ion in the solution. All the activity was adsorbed to the alumina, which was filtered off and eluted with 130 cc. of $M/15$ Na_2HPO_4 solution. Upon dialysis for 2 days against redistilled water (Cu^{++} -free) at pH 6.5–7.0, an amorphous dark colored protein-like precipitate was observed in the dialyzing bag. This precipitate was filtered off by means of a porcelain Gooch filter and found to have a brilliant bluish-green color. The filtrate was active but contained also most of the non-active protein as shown by the fact that the activity per milligram dry weight was low; i.e., 141 units per mg. The bluish-green precipitate was dissolved in 50 cc. of (Cu^{++} -free) 0.1 M Na_2HPO_4 (see preparation of Cu^{++} -free buffer solutions in legend of Fig. 1) to yield a highly active pale blue solution, containing 1970 units of enzyme activity per cc. and about 4.5 γ of Cu per cc. The blue color was comparable to that of a $Cu(NO_3)_2$ solution containing 2500 γ Cu^{++} per cc. The degree of purification effected by the precipitation during dialysis is shown by the fact that the blue solution had an activity of 1060 units per mg. dry weight. The pale blue solution contained a total of 88,500 units for a yield of 1.9 per cent of the original press juice activity. It is significant to note that these activity measurements were made in the manner prescribed by Lovett-Janison and Nelson (10). The dry weight and copper data on this solution, referred to as No. 15-OI in Table I, were obtained by further dialysis of a small sample for 2 days at pH 7.5 against frequent changes of redistilled Cu^{++} -free water. The dry weights of all solutions were determined by the method described by Lutz and Nelson (12), with the following modification; it was found unnecessary to seal off the dry weight bulbs after complete evaporation of the protein solution, because of the non-hygroscopic nature of this protein.

Copper Determinations

The method used for the copper determinations was that of Warburg and Krebs (13). To insure no contamination by extraneous copper, all samples were dialyzed, before making the copper determination, against distilled water which had been redistilled in Pyrex glass vessels.

Determination of Peroxidase Content of Purified Ascorbic Acid Oxidase

The method used for the determination of peroxidase was that of Balls and Hale (14). The purified oxidase preparation 15-OI was found to contain 0.000008 units of peroxidase per unit of ascorbic acid oxidase which compares favorably with a ratio of 0.0000078 obtained by Lovett-Janison and Nelson (10). The ratio in the crude squash juice was about 0.0022. The

preparation can be considered to be peroxidase-free, in view of the fact that in dilutions used to determine the ascorbic acid oxidase activity no peroxidase activity could be detected. Numerous authors (4, 10, 15) have reported that peroxidase activity accompanies ascorbic acid oxidase in their preparations; nevertheless the latter activity is independent of the former (15).

Determination of Catalase Content of Purified Ascorbic Acid Oxidase

Huszák (4) has reported a high concentration of catalase activity in his partially purified ascorbic acid oxidase preparation. However, the catalase activity in preparation 15-OI was found to be small. The method used for the determination of catalase activity was that of von Euler and Josephson (16).

TABLE I
Comparison of Several Ascorbic Acid Oxidase Preparations

Preparation No.	Concentration units/cc.	Units/ γ Cu	Per cent Cu	Units/mg. dry weight
14-3A1.....	594	456	0.11	510
14-IIB3B2-A.....	675	407	0.15	592
14-IIB3C2.....	600	400	0.15	588
11-L.J.*.....	531	406	0.15	612
12-L.J.*.....	516	490	0.15	631
15-OI.....	1970	450	0.24	1060
		Ave..... $428 \pm 25\ddagger$		
15-OI§.....	3125	715	0.24	1680

* Best preparations obtained by Lovett-Janison and Nelson.

‡ Lovett-Janison and Nelson preparations and the modified value (715) are not included in the calculation of the average.

§ Activity measured after modified dilution procedure.

A Kat. f. value of 204 was obtained when the catalase activity was measured at an enzyme dilution of 1:25 ($k_0 = 0.016$; dry weight = 1.86 mg. per cc.). No catalase activity was detectable in preparation 15-OI when it was diluted sufficiently to determine ascorbic acid oxidase activity.

Comparison of Ascorbic Acid Oxidase Preparations

Several ascorbic acid oxidase preparations were made by the Lovett-Janison and Nelson procedure (10). The data showing the relative purity of each preparation are given in Table I. The two best preparations of Lovett-Janison and Nelson are included for comparison. The proportionality of enzyme activity to copper content as shown in Table I (428 ± 25 units per γ of copper) was obtained when the activities were measured under the conditions described by the above workers (10). This value is in excellent agreement with their original value; i.e., 430 ± 30 units per γ of copper.

For reasons which will become apparent below, it was deemed advisable to alter slightly the conditions of activity measurement from those originally described (10) since the altered procedure for measurement gave a considerably higher activity for a given amount of the enzyme. This alteration consisted of making up the enzyme dilution with a dilute solution of the inert protein gelatin rather than adding the water-diluted enzyme to the gelatin at the initiation of the measurement. The increase in activity effected by this modified dilution procedure can be seen by inspection of the values in the bottom line of Table I.

It was found that these highly purified enzyme solutions could be stored in the refrigerator for several months with little or no loss of activity, provided the enzyme concentration was in excess of 1 mg. per cc., the solution was sterilized with a few drops of highly purified toluene, and the solution was kept about 0.1 M in Na_2HPO_4 . Such preparations, if dialyzed and stored in the absence of Na_2HPO_4 , were unstable in that they showed a tendency to precipitate the activity in the form of an amorphous greenish-blue protein.

The Use of Gelatin in Ascorbic Acid Oxidase Activity Measurements

Adams and Nelson (17) observed that the addition of the inert protein gelatin to the reaction mixture, when measuring the activity of the enzyme tyrosinase, furnished protection to the enzyme against inactivation. Lovett-Janison and Nelson made the same observation in connection with the ascorbic acid-ascorbic acid oxidase system. As a consequence, they recommended that during the activity measurement of ascorbic acid oxidase, 5 mg. of gelatin be placed in the main part of the Warburg respirometer flask, to stabilize the enzyme against inactivation during the oxidation of the ascorbic acid. Later studies by Miller and Dawson (18) on tyrosinase systems, showed that the protective action of gelatin was variable, depending on the type of enzyme and the environmental conditions during measurement. As a consequence of these findings, it seemed advisable to study in more detail the effect of the inert protein gelatin on the activity and inactivation of the highly purified ascorbic acid oxidase preparation 15-OI.

The data given in Fig. 1 show the effect of gelatin on the action of highly purified ascorbic acid oxidase during the aerobic oxidation of ascorbic acid. The experiments were performed at pH 5.6 using the same amount of enzyme and ascorbic acid in each case but in one of the three experiments no gelatin was present in the reaction system. For further experimental details see the legend of the figure. As can be seen from curve I of this figure, when no gelatin is present in the reaction system, the enzyme becomes inactivated very rapidly and the enzymatic oxidation ceases before the ascorbic acid is completely oxidized. The reproducibility of the activity measurements (rate of oxygen uptake per minute) is poor without the use of gelatin; comparable manometer

readings in two simultaneous runs varying as much as 20 to 25 per cent from one another. It is of interest to note (curve II) that with this preparation of the enzyme little or no protective action was observed when gelatin was used in the reaction mixture in the way prescribed by Lovett-Janison and Nelson—namely, the diluted enzyme is in the side arm during the temperature equilibration period and is then added to the reaction mixture containing gelatin at zero time. In the case of curve III, the reaction mixture contained the same amount of enzyme and less gelatin than in the case of curve II. However, the enzyme dilution was made with a dilute gelatin solution, rather than with water. Thus in this case (curve III) the enzyme was in a protein environment at all times during the process of dilution and temperature equilibration. A comparison of the initial rates of oxidation (curve III/curve I or II = 2.5/1.6 units in reaction flask) makes it apparent that much of the gelatin effect is one of protection against loss of enzyme activity occurring prior to the activity measurements; *i.e.*, the period of time between making the dilution and starting the measurement. It was found that this method of diluting the enzyme with gelatin did not change the range of concentration of enzyme over which there is a direct proportionality between rate of oxidation of ascorbic acid and the amount of enzyme used in the reaction flask. The most suitable range for measuring the activity was found to be from about 1 to 2.5 units of enzyme in the reaction flask under optimum conditions of pH and substrate concentration.

The loss of enzyme activity, when highly purified enzyme preparations are highly diluted, has been observed in several systems. Saul and Nelson (19) noticed a loss in activity when highly purified yeast invertase preparations were diluted. This abnormal decrease in activity almost vanished, when a little gelatin was added to the enzyme solution, either before or after dilution. Adams and Nelson (17) found, with highly purified tyrosinase preparations, a similar loss of activity upon dilution which was apparently reversed by using gelatin in the reaction system during the activity measurements. In the case of the highly purified preparation of ascorbic acid oxidase (15-OI) one notes that whereas a marked increase in activity is obtained when small amounts of gelatin are added to the enzyme during dilution, no such increase is observed when the diluted ascorbic acid oxidase is added at zero time to the reaction mixture containing gelatin. In other words, apparently gelatin cannot reverse the loss of enzyme activity occurring during the 15 minute temperature equilibration period. Experiments designed to learn more of this phenomenon were carried out and the results are discussed below.

To determine the effect of gelatin concentration, experiments were performed in which the concentration of gelatin in the 1 cc. of enzyme added to the reaction mixture was increased by several increments from zero to 5.5 mg. of gelatin per cc. The results are shown in Fig. 2. It will be seen that the rate of oxidation of ascorbic acid for a given amount of the enzyme-protein rapidly increases with

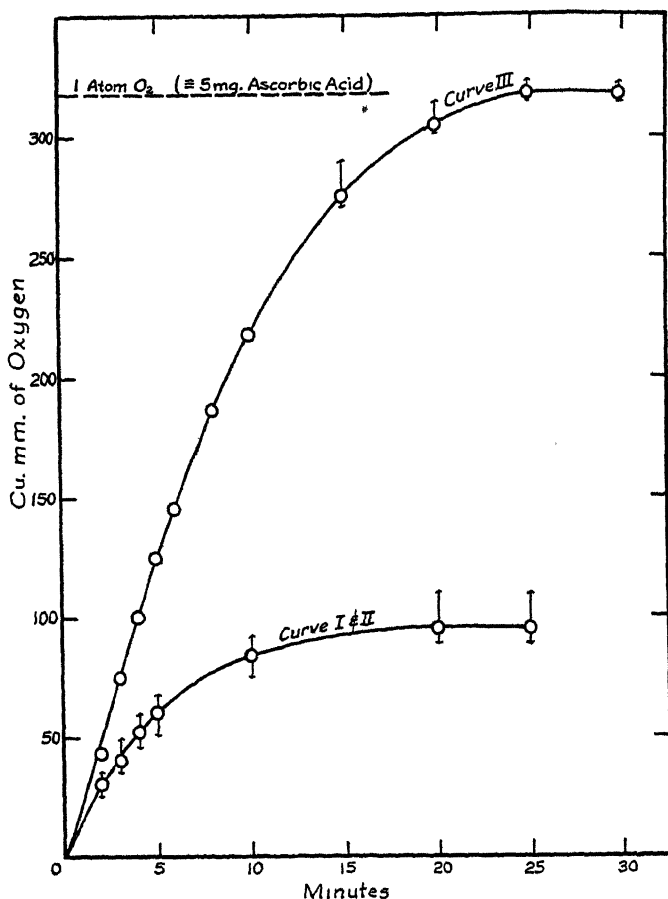


FIG. 1. Curves showing the effect of gelatin on the activity and inactivation of highly purified ascorbic acid oxidase.

Curve I. Reaction Mixture.—1 cc. of enzyme solution 15-OI (diluted 1:1250 with water as described below) placed in side arm of 50 cc. Warburg respirometer flask. Main part of flask contained 2 cc. of 0.1 M citric acid-0.2 M Na_2HPO_4 buffer, pH 5.7, 1 cc. of *L*-ascorbic acid solution (5 mg. per cc.) made up freshly each day in Cu^{++} -free water, and water to make a total reaction volume of 8 cc. pH of reaction mixture 5.6. Generally at least five Warburg manometers set up in identical fashion for each experiment. The flasks and contents were equilibrated in the thermostat at 25°C. for 15 minutes before initiating the reaction. Manometers were not shaken during this period in order to minimize surface denaturation of the enzyme-protein. During the run, the manometers were shaken at a rate of 120 oscillations per minute. Initial reading of oxygen uptake made at 2 minutes.

Curve II. Reaction Mixture.—Same as curve I, except 1 cc. of gelatin solution (5 mg.) substituted for 1 cc. of water in main reaction compartment.

increase in gelatin concentration up to 0.05 mg. of gelatin in the 1 cc. of enzyme. Very little difference in the initial rate of oxidation is effected by the use of more gelatin, although the optimum per cubic centimeter of enzyme appears to be in the vicinity of 0.5 mg. of gelatin. With much larger amounts of gelatin per cubic centimeter of enzyme, such as 5.5 mg., the initial rate of oxidation is a little lower and the reaction course is characterized by an initial lag period; *i.e.*, probably such large amounts of gelatin affect the initial enzyme-substrate relationship. In all subsequent work with this enzyme preparation in which gelatin was added during the enzyme dilution, the optimum concentration of 0.5 mg. of gelatin per cc. of enzyme solution was used.

The loss in activity of the enzyme, occurring when the enzyme is allowed to stand for a period in solutions of very low concentration, is undoubtedly due to some type of denaturation or unfolding of the enzyme-protein into less active forms. Apparently the gelatin exerts its effect by hindering this type of change of the enzyme-protein. To throw more light on this phenomenon of an inert protein protecting a highly active protein in this way, and to get some estimate of the time factor involved, the following experiments were performed. Varying amounts of time were allowed to elapse between making the final dilution and measuring its activity. During this time the enzyme was maintained at approximately 25°C. Two series of dilutions were set up; both to a final ratio of 1:1250. In one series the enzyme was diluted using only water, and in the other using gelatin solution so that the final concentration of gelatin was 0.5

Curve III. Reaction Mixture.—Enzyme solution diluted from subdilution (see below) as follows: 1 cc. of gelatin (5 mg. per cc.) and 1 cc. of subdilution diluted to 10 cc. with refrigerated water. 1 cc. of this dilution placed in side arm of flask; all other reactants as in curve I.

Diluting the Enzyme.—The enzyme dilution (1:1250) was made as follows: 1 cc. of the enzyme solution 15-OI (see Table I) was diluted to 25 cc. with water. This dilution could be used as a master dilution without loss of activity for a period of about a week. 1 cc. of this master dilution was next diluted to 5 cc. with water. This subdilution was found to be stable for several hours. The final dilution was made by diluting 1 cc. of the subdilution to 10 cc. All dilutions were made with Cu^{++} -free water kept at refrigerator temperature (about 2–5°C.) and were immediately stored in the refrigerator until used.

Other Details.—The buffer was made from citric acid and Na_2HPO_4 , each thrice recrystallized from distilled water which had been redistilled from all-glass apparatus. All Warburg flasks were cleaned with fresh dichromate cleaning mixture, rinsed with distilled water, and steamed out for 15 minutes prior to use. One flask containing all reactants except enzyme, was used as a blank in all runs. This served as a barometric control and also as a blank for ascorbic acid oxidation not due to enzymic oxidation. The latter was always found to be negligible during the time of reaction when the above cleaning precautions were observed. Arrows on the individual points of the curves represent maximum deviations from the average of several runs.

mg. per cc. of enzyme. The results are shown in Table II. It is apparent that the loss in activity is a function of time after making the final dilution, and that

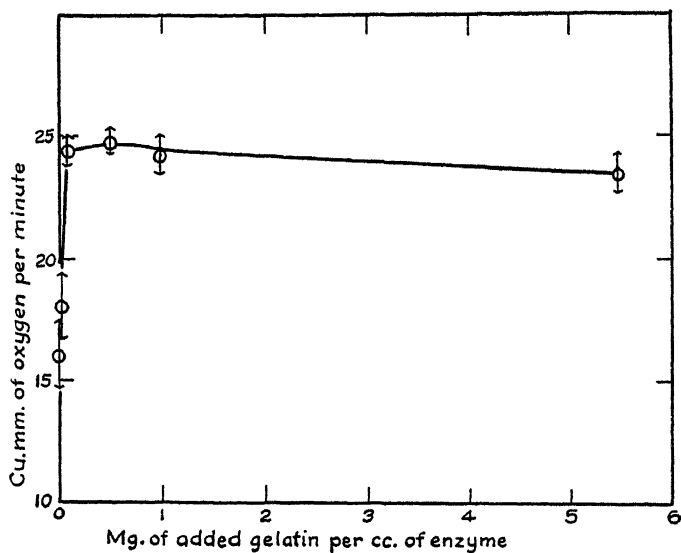


FIG. 2. Showing the effect of gelatin concentration on the initial rate of oxidation of ascorbic acid by a given amount of highly purified ascorbic acid oxidase. Gelatin added during dilution of enzyme.

1 cc. of enzyme (15-OI) diluted 1:1250 used for each rate determination. In making up the dilution, 1 cc. of the oxidase subdilution (described in legend of Fig. 1) was mixed with appropriate amounts of gelatin and diluted to a volume of 10 cc. with water kept at refrigerator temperature. In each rate determination 1 cc. of the gelatin-enzyme mixture was added from the side arm of the flask to initiate the reaction. Ascorbic acid, buffer, and other experimental details as described in legend of Fig. 1. The values of oxygen uptake per minute shown in the figure were obtained for the gelatin concentrations in excess of 0.5 mg. per cc. from the linear portion of curves showing the oxygen uptake as a function of time. In these cases little inactivation of the enzyme was evident within the first 5 minutes. For the lower concentrations of gelatin it was necessary to take the initial 2 minute reading as the initial rate of oxygen uptake. Each experimental point is the average of at least five determinations. Average deviations are represented by the arrows on each point.

the gelatin merely slows down the rate of denaturation or unfolding of the enzyme-protein.

pH-Optimum of Highly Purified Ascorbic Acid Oxidase

Various pH optima of ascorbic acid oxidase activity, measured in different buffer systems and with enzymes from various sources, have been reported by

a number of investigators. Using a citrate-phosphate buffer and a cucumber oxidase, Tauber, Kleiner, and Mishkind (3) reported an optimum lying between pH 5.56 and 5.93; Srinivasan (20) using drumstick oxidase reported pH 4.6 — 5.6, and Ghosh and Guha (21) reported a value of pH 5.6 for white gourd oxidase. In acetate buffer solutions, pH optimum values lying between 5.0 and 6.6 have been reported (3, 20, 21). While there seems to be little agreement in acetate buffers, all workers are fairly well agreed that in citrate-phosphate systems the optimum lies near pH 5.6 regardless of the source of the enzyme. This value has been substantiated using the highly purified summer squash oxidase (15-OI) (see Fig. 3). Since Steinman and Dawson (22)

TABLE II

Showing the Protective Effect of Gelatin upon the Stability of Diluted Ascorbic Acid Oxidase

Elapsed time between making dilution and initiating the reaction	Enzyme diluted* in water	Enzyme diluted in gelatin solution
	Activity/cc.	Activity/cc.
<i>min.</i>		
6	2.40 \pm 0.10 units	2.45 \pm 0.05 units
10	1.90 \pm 0.10 "	2.45 \pm 0.05 "
21	1.70 \pm 0.15 "	2.45 \pm 0.05 "
30	1.55 \pm 0.05 "	2.20 \pm 0.10 "
60	1.20 \pm 0.10 "	2.00 \pm 0.20 "

Rate determinations made using 1 cc. of enzyme (15-OI) diluted 1:1250 as described in legend of Fig. 1, (curves I and III) except that the freshly diluted enzyme was not placed in side arm of flask until after the 15 minute temperature equilibration period. Flasks were then quickly returned to thermostat and the reaction was initiated at 6 minutes, 10 minutes, etc., from the time the enzyme had been diluted. Room temperature about 25°C. The above activity values are the average of at least three determinations. Those in the gelatin series are based on linear portion of reaction course, whereas those in other (no gelatin) series are based on first 2 minute reading.

* No gelatin whatever added to Warburg flask.

have shown that the auto-oxidation of ascorbic acid in acetate buffers is considerable while in citrate buffer systems it is negligible, only the latter were used in this investigation. Although gelatin, used in the manner previously described, was found to decrease somewhat the sharpness of the pH optimum, the position of the optimum was not altered (compare curves I and II, Fig. 3). When the concentration of substrate was doubled (from 2.5 to 5 mg. of ascorbic acid in 8 cc. of reaction volume), no effect could be observed on the rate of oxidation or on the position or character of the pH optimum when the enzyme was diluted so as to contain 0.5 mg. of gelatin per cc.

Substrate Optimum Studies

Tauber, Kleiner, and Mishkind (3) have studied the effect of substrate concentration on the activity of cucumber ascorbic acid oxidase over a range of

1 to 5 mg. of substrate in a 7 cc. reaction volume and found that the activity of cucumber ascorbic acid oxidase was independent of the substrate concentration. Srinivasan (20) and Ebihara (7), using about the same range of variation of ascorbic acid, came to the same conclusion. The effect of larger concentrations

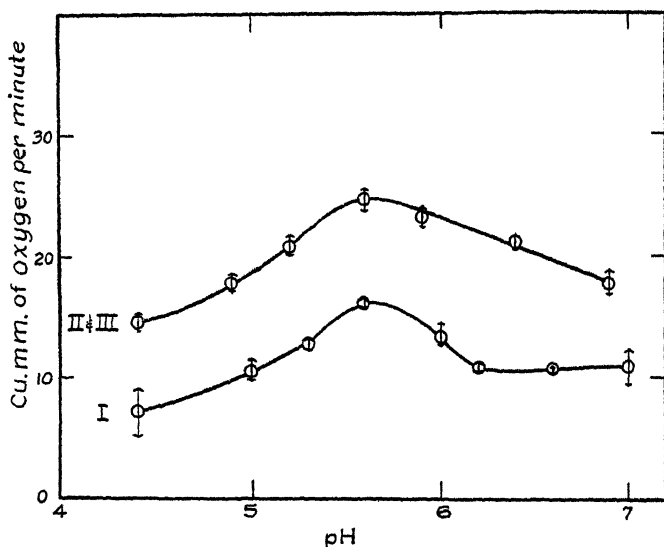


FIG. 3. Showing the effect of gelatin on the pH optimum of highly purified ascorbic acid oxidase.

Curve I. No Gelatin.—1 cc. of enzyme (15-OI) diluted (1:1250) as described in legend of Fig. 1. The 8.0 cc. reaction mixture contained 2.5 mg. of ascorbic acid and 2 cc. of 0.1 M citrate-0.2 M phosphate buffer (pH of mixture as shown). Rate measurements made as described in legend of Fig. 1.

Curve II. Gelatin.—1 cc. of enzyme (15-OI) diluted (1:1250) with gelatin as described in legend of Fig. 1 (curve III) (0.5 mg. gelatin per cc. enzyme). Ascorbic acid and buffer as in curve I.

Curve III.—Same as curve II except 5 mg. of ascorbic acid used.

Each experimental point the average of at least three determinations. Average deviation represented by arrows on each point.

of substrate on the enzyme apparently has not been previously studied. As will be seen from Fig. 4, the effect of varying the initial substrate concentration is somewhat more evident in the absence of gelatin than in its presence. When no gelatin is present in the system, (curve I) a significant decrease in the original rate of oxidation is effected by using amounts of ascorbic acid in excess of 5 mg. per 8 cc. reaction volume. However, when gelatin is present (curve II) there is little difference in activity caused by a variation in ascorbic acid from 2.5 to 10 mg. The results indicate that although purified ascorbic acid oxidase has

no sharp substrate optimum, activity measurements by the usual respirometer method should be made with about 5 mg. of ascorbic acid per 8 cc. reaction volume. They also indicate how inert protein material, such as is present in impure enzyme preparations, may tend to mask substrate enzyme relationships.

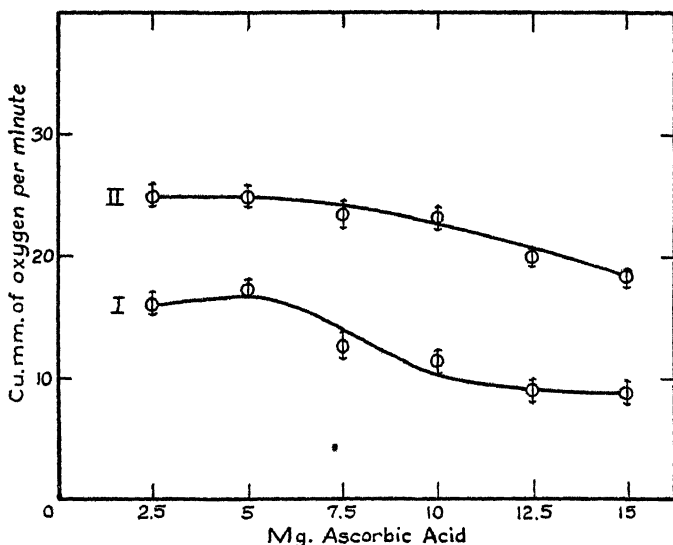


FIG. 4. Showing the effect of original substrate concentration on the activity of highly purified ascorbic acid oxidase.

Curve I. No Gelatin.—1 cc. of enzyme (15-OI) diluted with water. Measurements made at pH 5.6 as described in legend of Fig. 1.

Curve II. Gelatin.—1 cc. of enzyme (15-OI) diluted with gelatin as described in legend of Fig. 1 (curve III). All other conditions as in curve I.

The authors wish to thank Mr. F. J. Boyce of the Atlantic Commission Company, New York, for supplying the summer crook-neck squash that made this study possible.

SUMMARY

1. A method is described for the preparation of a highly purified ascorbic acid oxidase containing 0.24 per cent copper.
2. Using comparable activity measurements, this oxidase is about one and a half times as active on a dry weight basis as the hitherto most highly purified preparation described by Lovett-Janison and Nelson. The latter contained 0.15 per cent copper.
3. The oxidase activity is proportional to the copper content and the proportionality factor is the same as that reported by Lovett-Janison and Nelson.

4. When dialyzed free of salt, the blue concentrated oxidase solutions precipitate a dark green-blue protein which carries the activity. This may be prevented by keeping the concentrated solutions about 0.1 M in Na_2HPO_4 .

5. When highly diluted for activity measurements the oxidase rapidly loses activity (irreversibly) previous to the measurement, unless the dilution is made with a dilute inert protein (gelatin) solution. Therefore activity values obtained using such gelatin-stabilized dilute solutions of the oxidase run considerably higher than values obtained by the Lovett-Janison and Nelson technique.

6. The effect of pH and substrate concentration on the activity of the purified oxidase in the presence and absence of inert protein was studied.

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ON THE INACTIVATION OF ASCORBIC ACID OXIDASE

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An outstanding characteristic of the enzymatic oxidation of ascorbic acid by highly purified preparations of ascorbic acid oxidase, is the pronounced inactivation of the enzyme that occurs during the course of the reaction (1). It is the purpose of this communication to present data showing that the enzyme inactivation occurring during the reaction, is due to causes which, for the purposes of discussion and clarification, may be divided into two categories: (1) Inactivation due to environmental conditions during the reaction; *i.e.*, pH, substrate concentration, dilution, rate of shaking, etc., and (2) inactivation due to some factor inherent in the ascorbic acid-ascorbic acid oxidase-oxygen reaction. The first type of inactivation may be minimized by the use of the inert protein gelatin in the enzyme dilution under a prescribed set of environmental conditions (1). The second type of inactivation is not minimized to any great extent by the use of added gelatin. The groundwork for a study of the latter type of inactivation of ascorbic acid oxidase was laid by Steinman and Dawson (2), when they reported that catalase, peroxidase, and methemoglobin markedly protect ascorbic acid oxidase against inactivation. Inactivation encountered under various environmental conditions will be discussed first.

Effect of Substrate Concentration on the Inactivation of Ascorbic Acid Oxidase

When no inert protein is present in the reaction system, the enzymatic oxidation of ascorbic acid is characterized by a continuous decrease in the rate of oxygen uptake, until eventually the rate becomes zero, even though a considerable amount of unoxidized ascorbic acid may still remain in the flask. That this phenomenon is due to inactivation of the enzyme is evidenced by the fact that the addition of more enzyme to the system after the rate has approached zero results in a sharp increase in the rate of oxidation as measured by oxygen uptake. The total amount of oxygen absorbed by the system by the time the rate of oxidation approaches zero, will henceforth be called the "inactivation total" and will be expressed in mm.³ oxygen absorbed. Table I shows how the inactivation total varies with the substrate concentration for a given amount of enzyme and how it is affected by the inert protein, gelatin.

The data as given in columns 2 and 3 of this table show that when no gelatin is present in the reaction system, the amount of work (extent of oxidation) that a given amount of purified enzyme can accomplish before becoming completely inactivated decreases with increase in original concentration of ascorbic acid.

When gelatin is a component of the system the same general effect of original ascorbic acid concentration on the inactivation total is observed, but the effect is less pronounced (columns 4 and 5 of the table). It should be noted that because of the manner of introducing the gelatin, the higher inactivation totals observed in the latter case are the result in large part of less inactivation of the enzyme occurring prior to the start of the reaction (1).

TABLE I

Showing the Effect of Substrate Concentration with a Given Amount of Enzyme upon the "Inactivation Totals" in the Presence and Absence of Gelatin at pH 5.6*

Amount of ascorbic acid in 8 cc. reaction volume	Without gelatin		With gelatin	
	Inactivation total	Per cent of initial AH_2 oxidized	Inactivation total	Per cent of initial AH_2 oxidized
mg.	mm. ³ O ₂		mm. ³ O ₂	
2.5	109 ±7	72	159 ±2	100
5.0	98 ±9	31	311 ±4	100
7.5	62 ±3	13	347 ±11	73
10.0	58 ±4	9	217 ±4	34
12.5	36 ±5	5	146 ±4	18
15.0	31 ±3	3	133 ±3	14

Without Gelatin.—Reaction mixtures, set-up, and measurements made as previously described.† 1 cc. of enzyme (15-OI) diluted (1:1250) with H₂O used.

Mixtures of buffer and varying amounts of ascorbic acid adjusted to pH 5.6 by using 2.0 cc. of citrate-phosphate buffers of varying original values of pH.

With Gelatin.—Reaction mixtures same as above, except that 1 cc. of enzyme (15-OI) was diluted (1:1250) with gelatin solution‡ (0.5 mg. gelatin in system).

* "Inactivation total" is a term representing the total amount of oxygen absorbed by the system by the time the rate of oxidation approaches zero; *i.e.*, amount of oxygen absorbed before inactivation of the enzyme becomes complete. Each experimental value is the average of at least three determinations.

† See legend of Fig. 1, curves I and III, previous communication (1).

Effect of pH on the Inactivation of Highly Purified Ascorbic Acid Oxidase

The total amount of oxygen absorbed by the system before the point of complete inactivation of the enzyme (the "inactivation total"), varies with change in pH of the system in much the same manner as the activity of the enzyme varies with pH. This is shown by the data as given in Table II. Inspection of the data obtained using no gelatin and 2.5 mg. of ascorbic acid (columns 2 and 3) shows that the enzyme does the most work before inactivation at pH 5.6, the same pH that is optimum for the initial rate of oxidation (1). The same point is illustrated by the data obtained using gelatin and 5 mg. of ascorbic acid (columns 6 and 7). With inert protein (gelatin) in the system, however, the variation with change in pH is not as marked. Inactivation totals are considerably higher when gelatin is present in the system. As pointed out earlier, in the

discussion of Table I, this is due in large part to the manner of introducing the gelatin during the dilution of the enzyme, *i.e.* less enzyme is inactivated prior to its use in the reaction and effectively, therefore, more enzyme is added to the system at zero time than in the case of the data shown in columns 2 and 3. For this reason the effect of pH on the inactivation of this particular amount of enzyme cannot be illustrated using 2.5 mg. of ascorbic acid with gelatin, since the ascorbic acid is completely oxidized at each pH (columns 3 and 4).

TABLE II

Showing the Effect of pH with a Given Amount of Enzyme upon the "Inactivation Totals" in the Presence and Absence of Gelatin

pH of the system	No gelatin		Gelatin in enzyme dilution			
	2.5 mg. AH ₂ = 159 mm. ³ O ₂		2.5 mg. AH ₂ = 159 mm. ³ O ₂		5 mg. AH ₂ = 318 mm. ³ O ₂	
	Inactivation total	Per cent of initial AH ₂ oxidized	Inactivation total	Per cent of initial AH ₂ oxidized	Inactivation total	Per cent of initial AH ₂ oxidized
	mm. ³ O ₂		mm. ³ O ₂		mm. ³ O ₂	
4.5	23 ± 9	14	157 ± 3	100	199 ± 4	62
5.0	74 ± 4	46	154 ± 4	100	238 ± 12	75
5.3	94 ± 5	59	152 ± 6	100	243 ± 3	76
5.6	109 ± 7	70	159 ± 2	100	311 ± 4	100
6.0	78 ± 4	49	156 ± 1	100	263 ± 8	83
6.2	65 ± 4	41	157 ± 2	100	284 ± 9	89
7.0	65 ± 3	41	153 ± 3	100	194 ± 19	61

No Gelatin (2.5 Mg. Ascorbic Acid).—Reaction mixtures, set-up, and measurements made as previously described.* 1 cc. of enzyme (15-OI) diluted (1:1250) with H₂O used.

Gelatin (2.5 Mg. Ascorbic Acid).—Reaction mixtures same as above except that 1 cc. of enzyme (15-OI) was diluted (1:1250) with gelatin solution.*

Gelatin (5 Mg. Ascorbic Acid).—Reaction mixtures same as directly above, except that 5 mg. ascorbic acid used instead of 2.5 mg.

Each experimental value is the average of at least three determinations.

* See legend of Fig. 1, curves I and III, previous communication (1).

Effect of Shaking on the Inactivation of Ascorbic Acid Oxidase during the Enzymatic Oxidation of Ascorbic Acid

It was found necessary to regulate the shaking of the Warburg respirometers during the enzymatic oxidation of ascorbic acid by the highly purified ascorbic acid oxidase, since the amount of oxygen taken up before inactivation of the enzyme occurred was found to be dependent upon the rate of shaking. The following data illustrate the point. When 1 cc. of the enzyme solution (15-OI) diluted 1:1250 with water was used to oxidize 2.5 mg. of ascorbic acid in a reaction volume of 8 cc. buffered to pH 5.6 at 25°C., the ascorbic acid was completely oxidized (158 mm.³ oxygen uptake) when the manometers were shaken slowly

at a rate of 40 oscillations per minute. When the manometers were shaken at 120 oscillations per minute the rate of oxidation increased, but the "inactivation total" was 109 ± 7 mm.³ oxygen, and at a rate of 150 oscillations per minute a total of only 79 ± 7 mm.³ of oxygen was taken up before complete inactivation of the enzyme took place. Since the rate of oxidation was not increased in the latter case, all studies were made while shaking the manometers at 120 oscillations per minute.

Effect of Enzyme Concentration upon Inactivation of Ascorbic Acid Oxidase

The data in Fig. 1 show that, under specified conditions of pH, substrate concentration, and gelatin concentration, the "inactivation totals" vary directly with the amount of enzyme used in the reaction system. Ludwig and Nelson (3) observed the same phenomena while studying the inactivation of the copper protein enzyme, tyrosinase. Curve I shows the relationship between enzyme and "inactivation totals" under optimal conditions of pH and substrate concentration for measuring activity (*i.e.*, 5 mg. of ascorbic acid and at pH 5.6), while curve II shows the relationship under non-optimal conditions in regard to the substrate (*i.e.*, 10 mg. of ascorbic acid and at pH 5.6). The proportionality of enzyme concentration to total oxygen uptake is still direct under the latter conditions, but the slope of the line is less, since the inactivation total for any given amount of enzyme decreases with increase in substrate concentration (see Table I).

Inactivation Due to Some Factor Inherent in the Ascorbic Acid-Ascorbic Acid Oxidase-Oxygen Reaction

It is evident from the foregoing discussion and the data as shown in Fig. 1 that even when the environmental conditions are made optimum for minimizing the inactivation of the enzyme occurring during the course of the reaction, serious inactivation of the ascorbic acid oxidase is still apparent. In fact, only a minor portion of the inactivation occurring during the course of the reaction appears to be affected by environmental conditions. The fact that under these optimum conditions the amount of ascorbic acid oxidized before complete inactivation is proportional to the amount of enzyme employed, strongly suggests that some product of the reaction is responsible for the major portion of the enzyme inactivation. That the main product of the reaction, dehydroascorbic acid, is not responsible for this inactivation was shown in the following way. Experiments were set up in the Warburg respirometer, so that in each case the reaction mixture contained at the start of the reaction varying amounts of dehydroascorbic acid in addition to 5.0 mg. of ascorbic acid. This was effected by placing 5 to 12.5 mg. of ascorbic acid in the flask, and adding an amount of *p*-quinone sufficient to oxidize to dehydroascorbic acid all of the ascorbic acid in excess of 5 mg. Enzyme was then added immediately to ini-

tiate the enzymatic oxidation of the 5 mg. of ascorbic acid remaining in the flask. The experiment was set up four times so that the ratio of ascorbic acid to

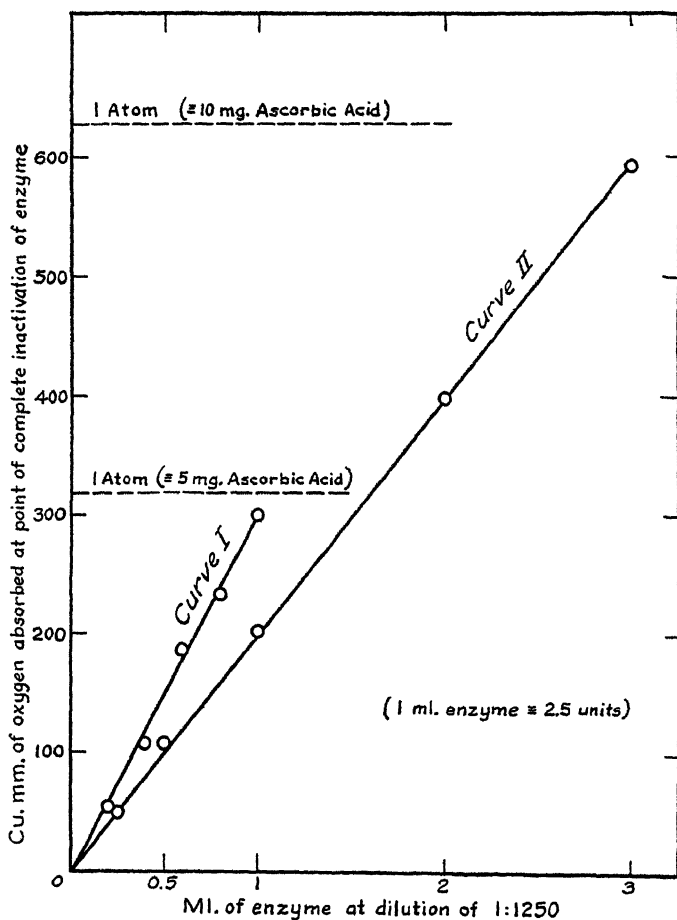


FIG. 1. Showing the dependence of inactivation on enzyme concentration at pH 5.6.

Curve I.—Reaction mixtures contained 5 mg. of ascorbic acid and 0.5 mg. gelatin. Set-up and measurements made as previously described (see legend of Fig. 1, curve III, previous communication (1)). 1 cc. of enzyme (15-OI) diluted (1:1250) with gelatin solution is equivalent to 2.5 units of activity.

Curve II. *Reaction Mixture.*—Same as for curve I, except 10 mg. of ascorbic acid.

dehydroascorbic acid was 5 mg./0 mg., 5 mg./2.5 mg., 5 mg./5 mg., and 5 mg./7.5 mg. In all four cases no difference in the rate of oxygen uptake was noticed and no significant difference in the total amount of oxygen absorbed before complete inactivation was found. Thus dehydroascorbic acid, or any

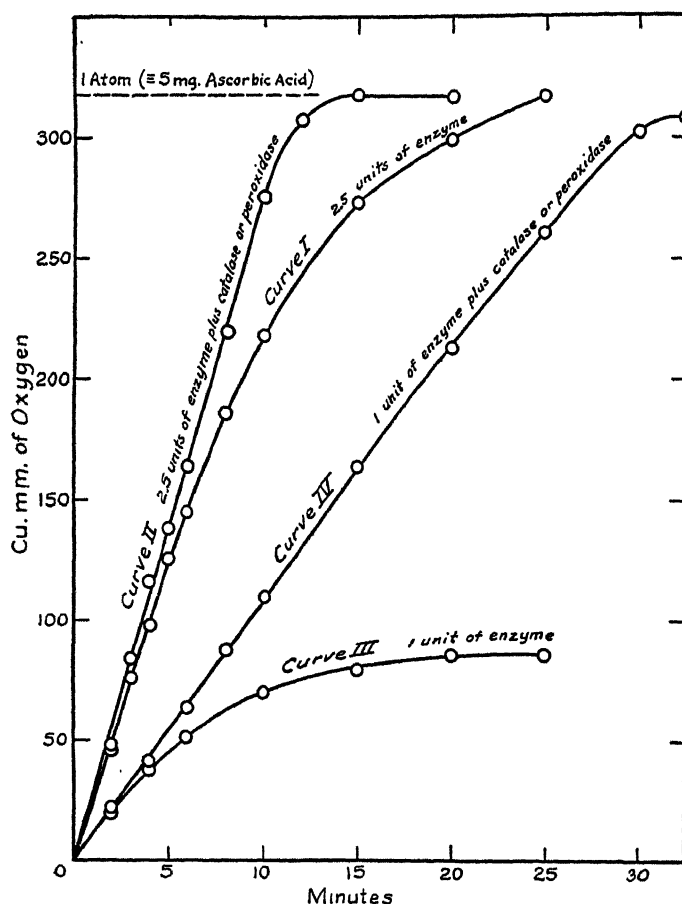


FIG. 2. Curves showing that the initial rate of oxidation of ascorbic acid by ascorbic acid oxidase is not affected by the presence of protecting agents such as catalase or peroxidase.

Curve I.—Reaction mixture contained 5 mg. of ascorbic acid and 0.5 mg. of gelatin (pH 5.6). Set-up, and measurements made as previously described (see Legend of Fig. 1, curve III, previous communication (1)). 1 cc. of enzyme (15-OI) diluted (1:1250) with gelatin solution was used (2.5 units).

Curve II.—Same as curve I except that 1 cc. of catalase or peroxidase (at a dilution of 1:1000 which contained 0.00217 mg. or 0.00275 mg. of protein respectively) was substituted for 1 cc. of water in reaction flask.

Curve III.—Same as curve I except that 1 unit of ascorbic acid oxidase was used; i.e., 1 cc. enzyme (15-OI) diluted (1:3125) with gelatin solution.

Curve IV.—Same as curve II except that 1 unit of ascorbic acid oxidase was used as in curve III.

Catalase Solution.—The catalase preparation was made and crystallized from beef

of its decomposition products, cannot be the factor causing the inactivation of ascorbic acid oxidase during the oxidation.

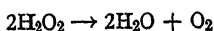
If one assumes that hydrogen peroxide is a product of the enzymatic oxidation of ascorbic acid,¹ as a number of investigators are inclined to assume (4-7), the inactivation of the ascorbic acid oxidase occurring during the reaction is readily explained. Dixon (11) has shown that when H_2O_2 is one of the products of an enzymatic oxidation, serious inactivation of the enzyme may occur during the reaction. Dixon studied the aerobic oxidation of hypoxanthine as catalyzed by xanthine oxidase, and found that the destruction of the enzyme could be prevented by the addition of catalase to the initial reaction mixture. With catalase present the initial rate of oxygen absorption by the system was con-

liver by E. S. Robinson in these laboratories. The method of preparation was that of Sumner and Dounce (13). It was recrystallized by one of us (W. H. P.) prior to use, following the suggestions for recrystallization offered by Sumner and Dounce. The activity of the undiluted catalase solution (also referred to in Table IV) was determined by the method of von Euler and Josephson (14) and found to have a Kat. f. value of 36,400. This value was obtained when the catalase solution was at a dilution of (1:2000). The dry weight of the undiluted solution was 2.17 mg. per cc. The iron content of the preparation was determined by the method of Saywell and Cunningham (15) with the following modification; *viz.*, concentrated nitric acid was substituted for perchloric acid during the wet-ashing process. The iron content was 0.106 per cent, each cubic centimeter of the undiluted catalase solution containing 2.3 γ Fe. The values for activity and iron content agree well with those of Sumner and Dounce (13) for their crystalline beef liver catalase; *e.g.*, 35,700 and 0.10 per cent Fe.

Peroxidase Solution.—The peroxidase preparation used in this work was made from horseradish roots by the method described by Elliott (16). The activity determination was performed according to the method of Balls and Hale (17) at an enzyme dilution of (1:1000). The preparation was found to have a P.Z. value of 680. The dry weight of the undiluted solution was 2.75 mg. per cc. The iron content was found to be 0.088 per cent, each cubic centimeter of the undiluted solution containing 2.5 γ Fe. The peroxidase solution at a dilution of (1:100) showed no catalase activity (failed to decompose any of 1.8 mg. of H_2O_2) over a period of 10 minutes. Solutions more concentrated than this showed slight catalase activity. The catalase solution above at a dilution of (1:100) under the same conditions completely decomposed 1.8 mg. of H_2O_2 in 20 minutes.

¹ The aerobic oxidation of ascorbic acid as catalyzed by cupric ion results in the formation of relatively large amounts of hydrogen peroxide (8-10, 2). However, there is no good evidence that hydrogen peroxide is a product of the oxidation as catalyzed by the copper-protein enzyme, ascorbic acid oxidase. In fact, the evidence is contradictory to the view that H_2O_2 *per se* is a product of the enzymatic oxidation (2). The same experimental methods which clearly indicate the presence of H_2O_2 in the ascorbic acid— Cu^{++} — O_2 system, even in the case where the amounts of H_2O_2 are very small, fail to detect H_2O_2 in the enzyme system.

siderably less, the reaction course showed little evidence of enzyme inactivation, and the oxygen uptake at complete oxidation of the hypoxanthine corresponded to one gram atom of oxygen instead of two gram atoms as obtained in the absence of catalase. These findings were confirmed by Keilin and Hartree (12) and the experiments have been repeated with the same results in this laboratory. These results indicate that hydrogen peroxide is a product of the enzymatic oxidation of hypoxanthine by xanthine oxidase. When catalase is present, the H_2O_2 is decomposed according to the equation



as rapidly as formed with a continuous return of oxygen to the system. The net result is a much lowered initial rate of oxygen uptake, protection of the enzyme, and a total uptake of one gram atom of oxygen instead of two.

When the same plan of attack was used on the ascorbic acid-ascorbic acid oxidase system by Steinman and Dawson (2), the results obtained were quite different. Although the enzyme was found to be markedly protected by small amounts of catalase in the system, the initial rate of oxygen uptake and the final volume of oxygen absorbed for complete oxidation of the ascorbic acid were unchanged. Fig. 2 shows the results of a similar series of experiments using the highly purified ascorbic acid oxidase preparation 15-OI. It is clear that when small amounts of catalase or peroxidase are present in the system the ascorbic acid oxidase is markedly protected against inactivation, and the phenomenon is not dependent upon the ascorbic acid oxidase concentration since the results are qualitatively the same whether 1 or 2.5 units of the enzyme are used. The initial rate of oxidation was not diminished by the presence of catalase, as would be expected if H_2O_2 were produced. Furthermore, the level of oxygen uptake attained in the protected systems is the same as that attained when sufficient quantity of ascorbic acid oxidase is used to completely oxidize the ascorbic acid.

Keilin and Hartree (12) have shown that the hydrogen peroxide produced in the enzymatic oxidation of hypoxanthine can cause the secondary oxidation of ethyl alcohol if catalase is present to catalyze this secondary reaction. This observation was confirmed and then applied to the catalase-protected ascorbic acid-ascorbic acid oxidase system. No secondary oxidation of ethyl alcohol could be detected, thereby indirectly giving further evidence that the ascorbic acid-ascorbic acid oxidase system does not produce H_2O_2 in the manner of the xanthine oxidase-hypoxanthine system.

Protective Action of Catalase and Peroxidase against the Inactivation of Ascorbic Acid Oxidase

A striking way of showing the degree of catalase or peroxidase protection against the inactivation of the ascorbic acid oxidase is to add to the system

varying amounts of either of these iron-porphyrin proteins, and then compare the amount of ascorbic acid oxidized (oxygen absorbed by the system) before the inactivation of the oxidase becomes complete. The results of a study of this sort are shown in Table III. Very similar results were obtained by substi-

TABLE III

Showing How the Oxidizing Capacity of a Given Amount of Highly Purified Ascorbic Acid Oxidase Is Increased with Increase in Catalase Added to the System

Experiment.....	I	II	III	IV	V	VI
Catalase added, mg.	0	0.000217	0.00217	0.0217	0.217	2.17
Approximate time before inactivation of the oxidase was complete....	10 min.	20 min.	2½ hrs.	3½ hrs.	5 hrs.	>8 hrs.
Ascorbic acid oxidized before inactivation, mg.....	1-1.5	2.0-2.5	18	31	46	>67

Experiment I.—Reaction mixture (buffered to pH 5.6) containing 15 mg. of ascorbic acid and 1 cc. of oxidase (15-OI) diluted (1:1250) with gelatin solution (0.5 mg. per cc.). Set-up and measurements made as previously described,* except that Barcroft differential manometers were used. The compensation flasks were made up the same as the reaction flasks except for oxidase.

Experiment II.—Same as I, except 1 cc. of catalase solution (see legend of Fig. 2) (dilution 1:10,000) was added to the initial reaction mixture in place of 1 cc. of water.

Experiment III.—Same as I, except 1 cc. of catalase solution (1:1,000) was added to the initial reaction mixture in place of 1 cc. of water. When all of the 15 mg. of ascorbic acid had been oxidized, a second 15 mg. portion of ascorbic acid was added and readings continued.

Experiment IV.—Same as I except 1 cc. of catalase solution (1:100) was added to the initial reaction mixture in place of 1 cc. of water. More ascorbic acid was added as soon as the previous amount had been oxidized.

Experiment V.—Same as I except 1 cc. of catalase solution (1:10) was added to the initial reaction mixture in place of 1 cc. of water. More ascorbic acid was added when necessary.

Experiment VI.—Same as I except 1 cc. of catalase (undiluted) was added. More ascorbic acid was added when necessary. The undiluted catalase solution had negligible ascorbic acid oxidase activity.

* See legend of Fig. 1, curve III, previous communication (1).

tuting peroxidase for catalase. The data show that the reaction between a given amount of the highly purified ascorbic acid oxidase and 15 mg. of ascorbic acid does not proceed far when the system is unprotected by catalase or peroxidase. However, if a very small amount of either catalase or peroxidase is added to the reaction mixture, this inactivation is prevented for a matter of hours and the amount of ascorbic acid oxidized before inactivation of the oxidase takes place is enormously increased.

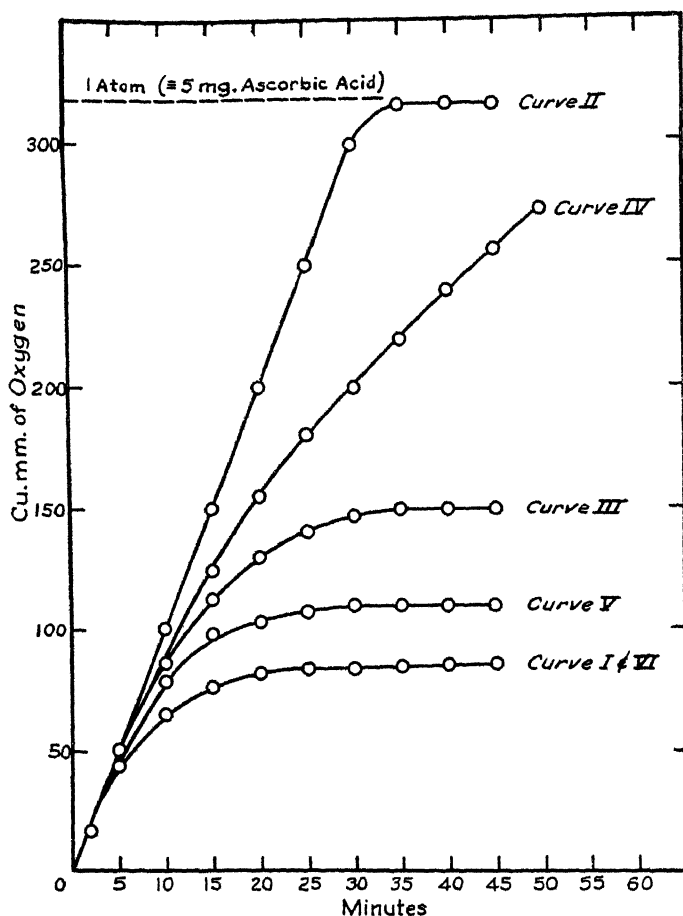


FIG. 3. Showing comparative effects of various proteins on the inactivation of ascorbic acid oxidase.

Curve I.—Base curve at pH 5.6 and 25°C. Original reaction mixture contained 5 mg. ascorbic acid and 1 unit of oxidase (15-OI) diluted with gelatin. Set-up and measurements made as previously described (see legend of Fig. 2, curve III).

Curves II to VI.—Same as curve I except that 1 cc. of water in original reaction mixture was substituted by 1 cc. of a solution containing the protective agents as shown in Table IV.

Other Details.—"Boiled" solutions were heated in a boiling water bath about 5 hours. "Boiled" catalase and methemoglobin solutions were in reality suspensions of the denatured protein. "Boiled" peroxidase solutions failed to precipitate out any denatured protein. The activity of both catalase and peroxidase solutions was completely nullified by the heating.

Cytochrome c Solution.—The cytochrome c solution was prepared according to

Effect of Porphyrin Proteins and Non-Porphyrin Proteins on the Inactivation of Ascorbic Acid Oxidase

The proteins which Steinman and Dawson (2) reported to be effective in preventing the inactivation of ascorbic acid oxidase were iron-porphyrins; *e.g.*, catalase, peroxidase, and methemoglobin. It seemed advisable, therefore, to study the effect of proteins other than iron-porphyrin proteins on this inactivation. Likewise, it seemed of interest to include the biologically active iron-porphyrin protein, cytochrome *c*, which from a structural viewpoint might be expected to play the same type of rôle as the above-mentioned iron-porphyrin proteins. Thus a comparison of the protective action of catalase, peroxidase, methemoglobin, cytochrome *c*, egg albumin, and gelatin was made under identical experimental conditions. The protein-free iron-porphyrin, hemin, was also tested for protective action.

The results are shown in Fig. 3. In all of the experiments represented in this figure, the reaction mixture contained 5 mg. of ascorbic acid and 1 unit of ascorbic acid oxidase (dilution was made with gelatin as previously described). In the case of curve I, nothing else was added to the system, and thus this curve shows the course of the enzymatic oxidation of the ascorbic acid in the absence of any protecting agents for the enzyme (except for the gelatin introduced during the dilution of the enzyme to stabilize it until the reaction was initiated). Curves II to VI show the effect of introducing varying amounts of the protective agents mentioned above and shown in Table IV. As can be seen from this table and reference to curves II, III, and V of Fig. 3, catalase and peroxidase

the method of Keilin and Hartree (18). The dry weight of the preparation was 1.55 mg. per cc. These authors report the iron content to be 0.34 per cent.

Methemoglobin Solution.—The methemoglobin solution was made from horse blood hemoglobin according to the method of Ferry and Green (19). The dry weight of the undiluted solution was 13.1 mg. per cc. Each cubic centimeter contained 38.3 γ of Fe, for an iron content of 0.29 per cent. The peroxidatic activity of this preparation at a dilution of (1:100) was slight, but not as strong as that of the peroxidase solution (legend of Fig. 2) at a dilution of (1:1000). The catalase activity of this preparation, at a dilution of (1:100) was sufficient to decompose 1.8 mg. of hydrogen peroxide in 50 minutes. The catalase solution at a dilution of (1:1000) decomposed an equal amount of hydrogen peroxide in 20 minutes.

Hemin.—Crystalline hemin was prepared by E. S. Robinson from ox hemoglobin according to the directions of Gatterman and Wieland (20). The solution of hemin as used above was made by dissolving the hemin crystals in a 0.01 per cent solution of NaOH and diluting with water to the desired hemin concentration. The percentage of Fe in hemin is 8.5. The amount of hemin as used above was 10 γ per cc.

Egg Albumin.—The egg albumin was prepared by S. R. Ames of these laboratories according to the method of Sørensen and Hoyrup (21). The dry weight of the preparation was 114.8 mg. per cc.

appear to be equally effective as protective agents against the inactivation of the ascorbic acid oxidase. In terms of iron, as little as 0.0025 microgram in the form of either active porphyrin protein enzyme, catalase, or peroxidase, completely protects the ascorbic acid oxidase against inactivation (curve II) and the effect of as little as 0.00023 microgram can be easily detected (curve III).

TABLE IV

Protective Agents Used in Experiments Shown in Curves of Fig. 3. All Values in Table Are Given in Micrograms

Curve	I	II	III	IV	V	VI
Catalase.....	0	2.17	0.217	0	0	217*
As Fe.....	0	0.0023	0.00023	0	0	0.23
		or	or			
Peroxidase.....	0	2.75	0.275	0	0	275*
As Fe.....	0	0.0025	0.00025	0	0	0.25
		or	or			
Methemoglobin.....	0	1310.0†	13.10	131.0†	1.31	0
As Fe.....	0	3.83	0.0383	0.383	0.00383	0
		or	or			
Hemin.....	0	10.0	1.0	0	0	0
As Fe.....	0	0.85	0.085	0	0	0
						or
Cytochrome c.....	0	0	0	0	0	1550
As Fe.....	0	0	0	0	0	5.3
						or
Gelatin.....	500	500	500	500	500	5500
						or
Egg albumin.....	0	0	0	0	0	5750

* Boiled solutions.

† Boiled or native solutions.

See legend of Fig. 3 for other details.

Larger amounts (ten and one hundred times as much) of these two enzymes, when introduced into the original system gave results identical to those shown in curve II.

No protective action was shown by solutions of catalase and peroxidase that had been boiled, even when relatively large amounts of the boiled solutions were used. This is shown by curve VI, which is superimposable on curve I. Since these solutions were boiled until they showed no catalase or peroxidase activity, it appears that catalase *activity* and peroxidase *activity* are necessary

attributes of these two iron-porphyrin proteins, to make them of value as protective agents against the reaction inactivation of ascorbic acid oxidase. In line with this, the two inert proteins, egg albumen and gelatin, were not found to be capable of protecting against the inactivation, even when large amounts were employed (curve VI). Likewise, the inability of cytochrome c to function as a protective agent is explicable on the same basis, for although cytochrome c, as an iron-porphyrin, is considered to be very similar in structure to both peroxidase and catalase, Keilin and Hartree (18) report that pure cytochrome c has no catalytic effect upon the decomposition of peroxides; *i.e.*, catalase or peroxidase activity. The fact that crystalline hemin itself is capable of exerting considerable protective ability (curves II and III) might be considered to be in line with this conclusion, since Haurowitz (22) has reported that crystalline hemin exhibits both catalase and peroxidase activity. The extent of these activities on an Fe basis is very much lower than for catalase or peroxidase, and it will be noticed that on the same basis the protective action of hemin is much lower.

The behavior of both native and "boiled" methemoglobin solutions in protecting against the inactivation of the oxidase is explicable in view of the results obtained with crystalline hemin. The methemoglobin solution used had both peroxidatic and catalatic activity in a slight degree. Whether these activities were noticeable because of the contamination of the methemoglobin solution by catalase and peroxidase or were due to a pseudoactivity of the hemin portion of the methemoglobin we did not attempt to prove. However, certain workers (23) do ascribe to hemoglobin a peroxidatic action in its own right. Since boiling completely destroyed both the catalytic activity of peroxidase and catalase as well as ruined their protective action on ascorbic acid oxidase, it does not appear that the heat-stable protective action of methemoglobin can be ascribed to contamination by peroxidase or catalase. Rather, it would seem that the protective action of "boiled" methemoglobin is due to a pseudo-catalase or pseudo-peroxidase activity of the hemin moiety of the molecule; an activity that is independent of "native" protein, just as in the case of the crystalline hemin.

All experiments using either catalase or peroxidase as the protecting agent were performed under two different conditions of ascorbic acid concentration. Catalase and peroxidase solutions (diluted 1:1000 and containing 0.00217 and 0.00275 mg. per cc. respectively) furnished complete protection against inactivation of the enzyme during the oxidation of 15 mg. of ascorbic acid as well as 5 mg. of ascorbic acid as is pictured in Fig. 3.

The Protective Action of Catalase and Peroxidase in the Presence of Iron-Porphyrin Enzyme Inhibitors

Sodium azide and hydroxylamine hydrochloride have been shown to be inhibitors of catalase and peroxidase activity (24-26). It seemed of value,

therefore, to study the effect of these inhibitors on the protection offered by these iron-porphyrin enzymes to ascorbic acid oxidase during the oxidation of ascorbic acid. The results of such a study are shown in the curves of Fig. 4. For comparison purposes curve I shows the oxidation of ascorbic acid by 1 unit of ascorbic acid oxidase in the absence of these protective agents, and curve II shows the oxidation course affected by the same amount of oxidase in a system containing excessive amounts of either catalase or peroxidase. Curve III shows that sodium azide (0.000125 M) has a marked inhibitory effect on ascorbic acid oxidase itself (compare with curve I) and increasing the concentration of azide to 0.000625 M did not change the reaction course any further. When the catalase-protected reaction system (curve II) was made 0.000125 M in respect to azide, an amount of azide sufficient to completely inhibit the catalase activity of the quantity of catalase used, as measured by the decomposition of H_2O_2 , curve IV was obtained. And when the same catalase system was made 0.000625 M in respect to sodium azide a curve superimposable on curve III was obtained.

Similar experiments with the peroxidase-protected system are shown by curves V and VI. Notice that the sodium azide does not decrease the protective action of peroxidase as much as it decreases the catalase protection. This is in agreement with the fact that azide is not as effective an inhibitory agent for peroxidase activity as it is for catalase activity (24). To obtain comparable inhibition in the peroxidase-protected system, it was found necessary to increase the azide concentration ten times over that used in the catalase-protected systems. These results with azide in the catalase- and peroxidase-protected systems indicate, as did the "boiled" catalase and boiled peroxidase experiments, that the protective action of these iron-porphyrin enzymes is closely associated with their catalase and peroxidase activity respectively, since inhibition of these activities decreases the protection offered by these enzymes in the ascorbic acid oxidase system.

The results of a similar study made using hydroxylamine hydrochloride as the inhibitory agent are shown in the curves of Fig. 5. Inspection of the data in this figure and its legend referring to the catalase-protected systems (curves II, IV, and III) shows that qualitatively the effect of hydroxylamine was the same as the effect of azide (compare with Fig. 4). The hydroxylamine, however, was not as effective as azide in nullifying the protective action of catalase. This is shown by the fact that in order to obtain comparable inhibition or nullification of the protective action of catalase, the system had to be made 0.018 M in hydroxylamine hydrochloride as compared to 0.000125 M in sodium azide. Experiments carried out to compare the effectiveness of these two agents in inhibiting the ability of catalase to decompose hydrogen peroxide illustrated qualitatively the same point; *i.e.*, azide is effective in much lower concentration than hydroxylamine. The latter was found to be rather ineffec-

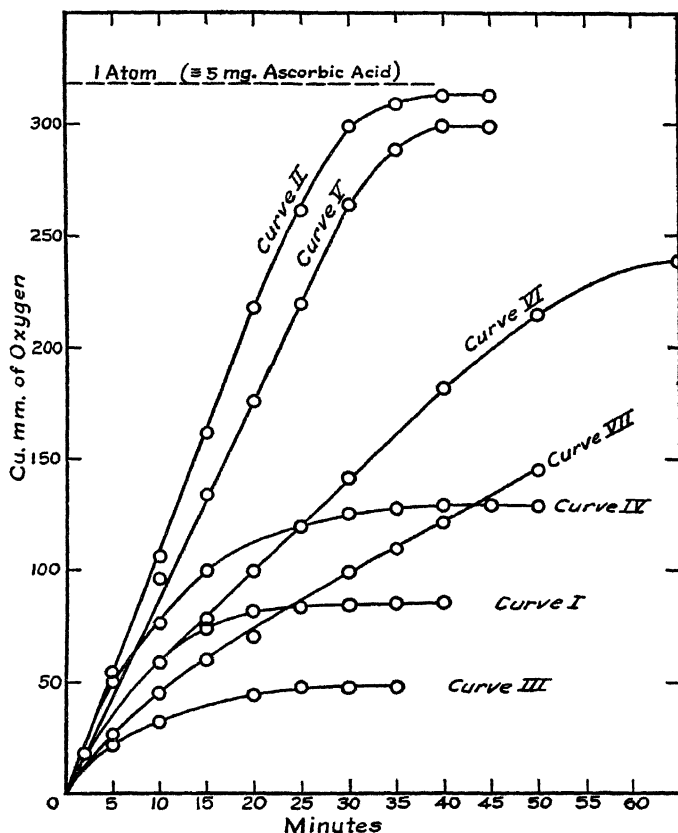


FIG. 4. Showing the effect of sodium azide on the catalase and peroxidase-protected oxidation of ascorbic acid by ascorbic acid oxidase.

Curve I.—Ascorbic acid and ascorbic acid oxidase. Reaction mixture same as that described in the legend of curve I, Fig. 3.

Curve II.—Ascorbic acid and ascorbic acid oxidase with catalase or peroxidase. Reaction mixture same as curve I above, except that system contained in addition 21.7 γ of catalase or 27.5 γ of peroxidase.

Curve III.—Ascorbic acid and ascorbic acid oxidase and sodium azide. Reaction mixture same as curve I, except that system was 0.000125 M with respect to sodium azide.

Curve IV.—Ascorbic acid and ascorbic acid oxidase and catalase and sodium azide. Reaction mixture same as curve II except that in addition to 21.7 γ of catalase the system was 0.000125 M with respect to sodium azide.

Curve V.—Ascorbic acid and ascorbic acid oxidase and peroxidase and sodium azide. Reaction mixture same as curve II, except that in addition to 27.5 γ peroxidase the system was 0.000125 M with respect to sodium azide.

Curve VI.—Ascorbic acid and ascorbic acid oxidase and peroxidase and sodium azide. Reaction mixture same as curve II, except that in addition to 27.5 γ peroxidase the system was 0.000625 M with respect to sodium azide.

Curve VII.—Ascorbic acid and ascorbic acid oxidase and peroxidase and sodium azide. Reaction mixture same as curve II, except that in addition to 27.5 γ peroxidase the system was 0.00125 M with respect to sodium azide.

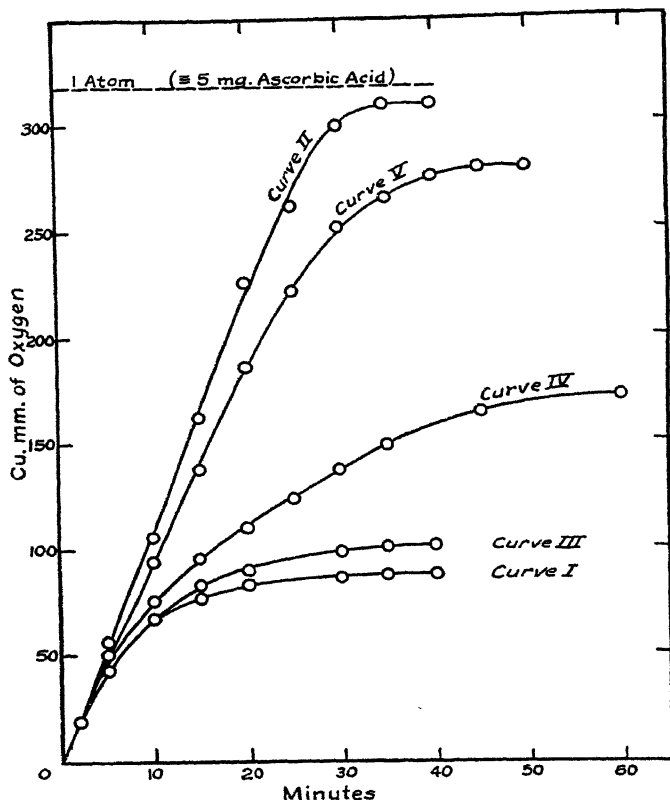


FIG. 5. Showing the effect of hydroxylamine hydrochloride upon the catalase- and peroxidase-protected oxidation of ascorbic acid by ascorbic acid oxidase.

Curve I.—Ascorbic acid and ascorbic acid oxidase. Reaction mixture same as that described in the legend of curve I, Fig. 3.

Curve II.—Ascorbic acid and ascorbic acid oxidase and catalase or peroxidase. Reaction mixture same as curve I, except that system also contained 21.7 γ catalase or 27.5 γ peroxidase.

Curve III.—Ascorbic acid and ascorbic acid oxidase and hydroxylamine hydrochloride. Reaction mixture same as curve I above, except that the system was 0.0018 M with respect to hydroxylamine hydrochloride; curve III also obtained when system was 0.018 M with respect to hydroxylamine hydrochloride. This also represents the oxidation of ascorbic acid by ascorbic acid oxidase when the system is 0.018 M with respect to hydroxylamine hydrochloride and also contains 21.7 γ of catalase.

Curve IV.—Ascorbic acid and ascorbic acid oxidase and catalase and hydroxylamine hydrochloride. Reaction mixture same as curve II above, (catalase), except that the system was also 0.0018 M with respect to hydroxylamine hydrochloride.

Curve V.—Ascorbic acid and ascorbic acid oxidase and peroxidase and hydroxylamine hydrochloride. Reaction mixture the same as curve II above (peroxidase), except that the system was either 0.0018 M or 0.018 M with respect to hydroxylamine hydrochloride. The same results were obtained using 0.131 mg. of methemoglobin instead of peroxidase with the same concentrations of hydroxylamine hydrochloride. Without the hydroxylamine this quantity of methemoglobin produced results as pictured in curve II.

tive as an inhibitor or nullifying agent in the peroxidase-protected and methemoglobin-protected systems (curve V).

DISCUSSION

The fact that in the ascorbic acid-ascorbic acid oxidase reaction, marked protection of the oxidase against inactivation is affected by introducing small amounts of either catalase or peroxidase to the system, and the fact that the protection is closely associated with the catalytic activity of these iron-porphyrin proteins, would lead one at first thought to attribute the inactivation to the formation of H_2O_2 as a reaction product of the enzymatic oxidation of ascorbic acid. This explanation, however, is untenable for the following reasons, as pointed out by Steinman and Dawson (2) and confirmed in this study: (1) Neither of these protective agents alters the initial rate of the reaction nor changes the volume of oxygen absorbed during complete oxidation of the ascorbic acid as measured manometrically. If H_2O_2 were being decomposed, oxygen would be returned to the system, thereby lowering the initial rate of oxygen uptake and the total volume of oxygen absorbed per milligram of ascorbic acid completely oxidized. (2) The peroxidase, even in ten times the concentration needed to show marked protective action, would not decompose H_2O_2 alone, or at any significant rate in the presence of ascorbic acid. (3) Methemoglobin and hemin, in concentrations that exhibited good protective action, showed only slight ability to decompose H_2O_2 .

Yet it is apparent, in view of the following facts, that the protective action of these iron-porphyrin proteins is in some way closely associated with their enzymatic activity. (1) Boiling the catalase and peroxidase preparations destroys their protective action as well as destroys their enzymatic activity. (2) Catalase and peroxidase inhibitors such as sodium azide and hydroxylamine hydrochloride also inhibit the protective ability of these enzymes. (3) Cytochrome c has no protective ability against the inactivation of the oxidase and likewise has no enzymatic action upon H_2O_2 . (4) Non-enzymatic proteins such as gelatin and egg albumin have no protective action on this type of inactivation.

One explanation of these seemingly contradictory facts suggests itself. That is, that the inactivation of the oxidase may be due to some precursor of hydrogen peroxide; *i.e.*, a "redox" form of oxygen having a transitory existence because of performing some intermediary function in the enzymatic oxidation of ascorbic acid. It would be assumed that catalase and peroxidase have the ability to destroy this precursor at a high rate as long as they are enzymatically active, and that such decomposition would not affect the oxygen uptake or oxygen totals for complete oxidation of the ascorbic acid. It would likewise be necessary to assume that hemin, and therefore native or denatured methemoglobin, can also decompose the precursor when present in sufficient concentration.

SUMMARY

1. In the absence of protective agents, highly purified ascorbic acid oxidase is rapidly inactivated during the enzymatic oxidation of ascorbic acid under optimum experimental conditions. This inactivation, called reaction inactivation to distinguish it from the loss in enzyme activity that frequently occurs in diluted solutions of the oxidase prior to the reaction, is indicated by incomplete oxidation of the ascorbic acid as measured by oxygen uptake; *i.e.*, "inactivation totals."

2. A minor portion of the reaction inactivation appears to be due to environmental factors such as rate of shaking of the manometers, pH of the system, substrate concentration, and oxidase concentration. The presence of inert protein (gelatin) in the system ameliorates the environmental inactivation to a considerable extent, and variation of the above factors in the presence of gelatin has much less effect on the inactivation totals than in the absence of gelatin.

3. A major portion of the reaction inactivation of the oxidase appears to be due to some factor inherent in the ascorbic acid-ascorbic acid oxidase-oxygen system, possibly a highly reactive "redox" form of oxygen other than H_2O_2 or H_2O . The inactivation cannot be attributed to dehydroascorbic acid, the oxidation product of ascorbic acid.

4. Small amounts of native catalase, native peroxidase, native or denatured methemoglobin, and hemin when added to the system, markedly protect the oxidase against inactivation. Cytochrome c has no such protective action. Likewise proteins such as egg albumin, gelatin, denatured catalase, or denatured peroxidase show no such protective action.

5. None of the protective agents mentioned above affect the initial rate of oxygen uptake or change the total oxygen absorbed for complete oxidation of the ascorbic acid, and hence do not act by removal of hydrogen peroxide, *per se*.

6. Sodium azide and hydroxylamine hydrochloride which inhibit catalase and peroxidase activity also inhibit the protective action of these iron-porphyrin enzymes.

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STUDIES ON THE ANOMALOUS VISCOSITY AND FLOW-BIREFRINGENCE OF PROTEIN SOLUTIONS

I. GENERAL BEHAVIOUR OF PROTEINS SUBJECTED TO SHEAR

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INTRODUCTION

The experiments to be described in this communication and in succeeding communications in the same series originated from a belief that further knowledge of the shapes of molecules, especially proteins, may throw light upon the nature of morphological and histological shapes, and the changes which they undergo. We were especially interested in the formation of the neural tube in the amphibian embryo, where this, the first, morphological change in development, consists of a tenfold lengthening of the cuboidal ectodermal cells and nuclei to form the cells of the neural plate. The question arises whether such morphological transformations may not depend upon, or be accompanied by, an increase in the number, size, or axial ratio, of anisometric protein particles present.

As far back as 1912 Koltzov suggested that protein particles of a highly anisometric character might have an important part to play in maintaining cell structure. At that early period his view could only be based upon logical deductions from the Emil Fischer polypeptide chain hypothesis of protein constitution. But by the time of his further review (Koltzov, 1928), several different lines of attack were combining to demonstrate the fundamental correctness of his ideas. The x-ray examination of the cellulose of plant cell walls (Sponsler, 1925; Sponsler and Dore, 1926) was already beginning to show the oriented state of the elongated cellulose micelles, and the similar studies of the fibre proteins of textile strands, hair, and the like (summarised in the book of Astbury, 1933) followed not long afterwards. About the same time, the work of von Muralt and Edsall (1930 *a*) on the principal protein of muscle tissue, the globulin myosin, in which the method of flow-birefringence was first employed in physiology, demonstrated that the micelles of this protein are in fact highly elongated, in accordance with the shape of the muscle cells themselves.

Numerous other methods have been used to investigate the shape of protein particles, notably (1) the Mie-Siedentopf effect (sparkling of the Tyndall beam when long particles are present) used by Szegvari (1923, 1924); (2) the Gans effect (depolarisation of the Tyndall beam) used by Wöhlisch and Belonoschkin (1936); (3) the Majorana effect (birefringence caused by orientation of particles

in electromagnetic fields) used by Bergholm and Björnsthåhl (1920); (4) the dielectric dispersion method of Oncley (1938); (5) the freezing speed method (Freundlich and Oppenheimer, 1925; Püllen, 1933); and (6) the calculations of Neurath (1939) from diffusion constants and relative viscosities. All these methods study particles which are at rest, or moving only very slowly. But there are also (7) the change of Tyndall beam intensity when the sol is sheared, if long particles are present (Dieselhorst, Freundlich, and Leonhardt, 1916); and (8) the anomalous dependence of viscosity on shear force (Rothlin, 1919). Perhaps the most promising approach at the present time is (8) the method of simultaneous measurement of anomalous viscosity and flow-birefringence in a modified form of the coaxial (Couette) viscosimeter. The theory of this method was first worked out by one of us with Robinson (see Robinson, 1939), who applied it to tobacco mosaic disease virus nucleoprotein¹ and other proteins. In the present work we have concentrated on the measurement of flow-birefringence and anomalous viscosity, as indications of molecular asymmetry.

On the whole subject of protein particle shape in relation to living organisation, a rather large literature has now grown up, and it cannot be reviewed in this place. The remarkable properties of polarity and symmetry which eggs and embryonic cells possess, the phenomena of determination of spatial axes of embryonic structures at different points in time, etc., etc., have led to the conception of a "cyto-skeleton" or framework of highly dynamic nature (*cf.* Peters, 1929; Needham, 1936, 1942; Seifriz, 1936; Frey-Wyssling, 1938). On approaching such possibilities for the first time, one is apt to be impressed by the very large difference of scale between the largest protein particle, however anisometric it may be, and the smallest histological form, but it must be remembered that readily breakable and re-formable chains may exist, the conditions for existence of which must include the shape of the ultimate particles of which they are composed. It is not perhaps irrelevant to refer, in this connection, to events at a higher level. In human technology we are aware of numerous instances where the final (morphological) form has been influenced by the nature of the building materials, *e.g.* the stockades of Saxon churches, the dependence of apsidal construction upon the use of bricks, and the functional possibilities permitted by steel and concrete. There are numerous indications that anisometric particles may exist in eggs, and embryonic, as well as adult, cells. Birefringence has long been known in amoeboid pseudopodia, filopodia, and axopodia (Engelmann, 1875, 1881; MacKinnon, 1909; Schmidt, 1937*a*), in chloroplasts (Weber, 1937), and in chromosomes (Nakamura, 1937; Kuwada and Nakamura, 1934)—see the books of Schmidt (1924, 1937*b*). Birefringent phases in echinoderm egg cytoplasm have been reported by Schmidt (1936) and Moore and Miller (1937). Pfeiffer (1936, 1937) observed optical anisotropy and true anomalous flow when naked cytoplasm is caused to pass at different pressures through a capillary tube. Pollister (1941) has described orientations of mitochondria in embryonic cells which suggest the orientation of elongated submicroscopic particles in the cytoplasm, while Hobson (1941) has demonstrated an orienta-

¹ Hereinafter referred to as TMD virus.

tion of molecules, both protein and lipoidal, in the intact chick embryo by the aid of polarisation microscopy. Egg proteins, too, have given indications of their anisometric character, *e.g.* avian ovoglobulin (Böhm and Signer, 1931), avian livetin (Needham and Robinson, 1937), and a fraction from the echinoderm egg (Mirsky, 1936).

No doubt the most striking instance of elongated particle shape in proteins is the case of the plant viruses, especially the TMD virus (Bawden, Pirie, Bernal, and Fankuchen, 1936; Bernal and Fankuchen, 1941) where the particles have an axial ratio of the order of 100. It has since been possible to obtain actual photographs of these elongated particles with the aid of the electron microscope (Kausche, Pfankuch, and Ruska, 1939). The tendency of elongated molecules to form liquid crystals, tactoids, etc., is well known, and is especially marked in the case of these viruses. The existence of paracrystalline phases within the living cell is therefore of much importance for cell architecture, as has often been pointed out (as by Przibram, 1926; Rinne, 1931; Needham, 1936). The spindle in cell division is probably a tactoid (Bernal, 1939). Moreover, important biological substances other than proteins show flow-birefringence and anomalous viscosity, *e.g.* the polymerised particles of sodium thymonucleate (Signer, Caspersson, and Hammarsten, 1938; Greenstein and Jentette, 1940*a*). The chromosomes themselves are built up on the chromonema thread (Muller, 1941) of nucleoprotein fibre molecules (Koltzov, 1928; Wrinch, 1936; Schultz, 1941) and are highly extensible, like myosin, though not fully elastic (Buck, 1942).

The biological significance of the phenomena to be reported in this series of papers may therefore be summarised as follows: We are concerned with: (1) the location of protein fractions showing elongated particle shape, and the participation of these in the architecture of the living cell, both embryonic and fully developed; (2) the mutual interactions of substrates and enzymes, when the latter are themselves elongated particles, involving changes, reversible or irreversible, in the configuration of the enzyme micelle; (3) the formation of the elongated molecules and micelles by the living cells—the processes by which they are “spun;” (4) the formation of microscopically visible fibres, as *e.g.* in connective tissue.

General Principles of the Methods

The first step towards a hydrodynamical treatment of the viscosity of colloidal solutions was the Einstein equation (Einstein, 1906, 1911), usually cited in the sterile form: $\eta = \eta_0(1 + F V)$ where η is the viscosity of the suspension, η_0 that of the dispersion medium, and F is a factor equal to 2.5 for spherical particles; without any description of its derivation. The equation was calculated for spherical particles large enough for Brownian motion to be negligible and for concentrations small enough to ensure absence of any sort of mutual interference. In a velocity gradient, the spheres rotate carrying with them a region of disturbed flow in the surrounding liquid dispersion medium. This dissipation of energy over and above that used in maintaining stream line flow,

results in an apparent overall viscosity of the suspension higher than that of the solvent. The viscosity of the solvent is unchanged; only its conditions of flow are affected by the presence of the particles.

When we consider anisometric particles, it is clear that their interference with normal stream line flow will be greater than that due to an equal volume of spherical particles. Further, it is clear from Fig. 1 that the maximum torque is exerted upon the particle when lying athwart the stream lines, and that the position of minimum dissipation is with the long axis of the particle in the direction of streaming. Earlier workers assumed that rod-shaped particles were orientated irreversibly in this direction by flow, but although this state is approached in certain aged sols in which the particle length is very great, continu-

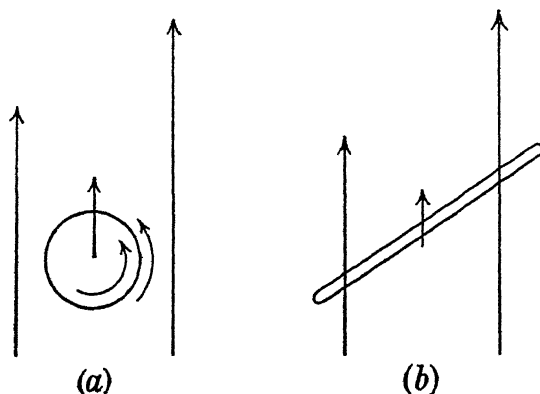


FIG. 1. Motion of particles in streaming flow. The length of arrows is proportional to streaming velocity.

ous disorientation also occurs owing to the finite cross-section of the rods and to Brownian motion.

Jeffery (1922) and Eisenschitz (1933) extended the hydrodynamical treatment to ellipsoidal particles assuming a steady precessional movement without irregularities due to Brownian motion. Jeffery determined the position of minimum dissipation of energy but his results are not in accord with the experimental results obtained from measurements of flow-birefringence. His position of equilibrium for minimum dissipation of energy is for the rods to be rotating about their long axes with these parallel to the axes of the (Couette) viscosimeter. Taylor (1923) confirmed this experimentally for particles with small axial ratio. Increasing the length of the particles, however, causes them to deviate from this position, and with increasing rate of shear, their plane of rotation approaches that shown in Fig. 1; *i.e.*, at right angles to the axis of the instrument (Guth, 1936). It may be noted that there will always be a very small vertical velocity gradient in the Couette apparatus which would explain

this difference of behaviour between very long thin fibrils and Taylor's ellipsoids with much smaller axial ratio; *e.g.*, 3.

The treatment most used, however, is a simple extension of the Einstein equation consisting of replacement of the V term by one which takes into account the larger effective volume of an anisometric particle rotating in the velocity gradient. Staudinger (1932) takes this effective volume to be a flat disc of diameter equal to the particle length and of thickness equal to the particle breadth. It would seem that an additional factor should be added for energy dissipated in the region of liquid in disturbed flow around the "disc" of each particle, but this factor will be small compared with that in the "disc" itself. Actually, of course, Staudinger's disc is an idealised picture of the integral of a number of motions each of which is a sector of such a disc but which are not all in one plane. Staudinger's attempt to justify his equation on hydrodynamical grounds by extension of the Einstein equation is obviously unsound but Huggins (1939) later derived an equation which reduced to Staudinger's for the special case of long *kinked* particles. Kuhn (1932) has also considered viscosity and flow-birefringence for cases where there is no preferred orientation of the particles, but he attributes the birefringence to the strain caused in a model particle by the pull on the two ends moving at different velocity. Although strain-birefringence may be found in gels under shear, orientation-birefringence has been established in so many cases that we must consider it in more detail. Boeder (1932) first discussed the theory which is outlined below and a number of other workers (Haller, 1932; Guth, 1936; Guth and Simha, 1936; Peterlin, 1939) have made important contributions, (review by Eirich, 1940).

The two actually occurring cases considered so far in this paper are: (*a*) anisometric particles so large that, even at low velocity gradients, orientation occurs with the long axis of the particles along the stream lines, *i.e.* the position of minimum dissipation of energy and therefore of minimum viscosity and of maximum birefringence. Many earlier workers implicitly assumed that flow-birefringence and apparent viscosity increased together whereas it is obvious that the two properties must move in the opposite sense with change of rate of shear, as it has been proved experimentally that they do (Lawrence, 1937; Robinson, 1939); (*b*) anisometric particles so small that realisable shear rates are insufficient to overcome the disorientation caused by Brownian motion, so that they progress with irregular precessional movements.

Consider now (*c*) the behaviour of anisometric particles of intermediate length in a velocity gradient. In the Couette viscosimeter, Fig. 1 (*b*) will represent a plane at right angles to the axis of the instrument. The rod-shaped particle lying athwart the velocity gradient is rotated since its two ends are moving with different velocities. The overall motion of the particle is therefore a precessional one—rotation plus linear motion along the stream lines.

The particle will turn until its long axis coincides with the stream lines. If its cross-sectional area is sufficiently small there will be no further couple exerted on it by the flowing liquid. Against this hydrodynamical orientation, however, is Brownian disorientation. If streaming were stopped at this point, disorientation by Brownian motion would follow. For a spherical particle, Einstein has given the equation:

$$\frac{A^2}{t} = \frac{RT}{4\pi N r^3 \eta}$$

where A is one-third of the square of the mean angle of rotation in time t , r is the radius of the spheres, η the viscosity of the liquid dispersion medium, and the other terms have their usual significance. It is clear that Brownian disorientation may be assessed as a rotatory diffusion constant, D , which is given by $D = \frac{RT}{8\pi\eta r^3}$ while for rods we get

$$D = \frac{3KT}{8\pi\eta} \left(\frac{\log l/r - 0.8}{(l/2)^3} \right)$$

(Burgers, 1938). A sol containing anisometric particles is therefore in a state of equilibrium between hydrodynamic orientation and Brownian disorientation. Orientation will increase with particle size (axial ratio) and with rate of shear. Disorientation will decrease with particle size (axial ratio) and with viscosity of the dispersion medium but will *increase* with rise of temperature on account of both increased heat motion of the particles and reduction of viscosity of the medium in which they rotate (Langmuir, 1938 *b*; Robinson, 1939; Lawrence, 1940).

When an anisometric particle is rotated by Brownian motion in a clockwise direction (Fig. 1), the velocity gradient will oppose its rotation. In the converse direction, the effects will be additive. The particles will therefore no longer have their minimum angular velocity in the stream line direction but in a position displaced from it in the clockwise sense. At low rates of shear, the particles will need to rotate through a considerable angle before the velocity gradient force balances the rotation diffusion one. At higher rates of shear this angle will fall. We see therefore that the angle of preferred orientation will vary with velocity gradient or rate of shear. This direction of orientation is denoted by the angle ψ . Fig. 2 shows the appearance of the field when a sol of anisometric particles is sheared in a coaxial cylinder viscosimeter and viewed along the axis by polarised light with Nicols crossed. The four black "brushes" mark the positions where the optic axes of the particles are parallel to the planes of polarisation of the Nicols. ψ is defined as the larger of the two angles made by the radius, on which the brush lies, with the planes of polarisation. Clearly it must lie between 45° and 90° .

At this stage, we must note that there are three different types of flow-birefringence. There is: (a) *Strain-birefringence* which is found in elastic gels (Kunitz, 1930) and in certain liquids at very high rates of shear (Clerk-Maxwell, 1874; Kundt, 1881; Umlauf, 1892; Vorländer and Walter, 1925). Stokes showed that this photoelastic effect arises from axes of pressure and

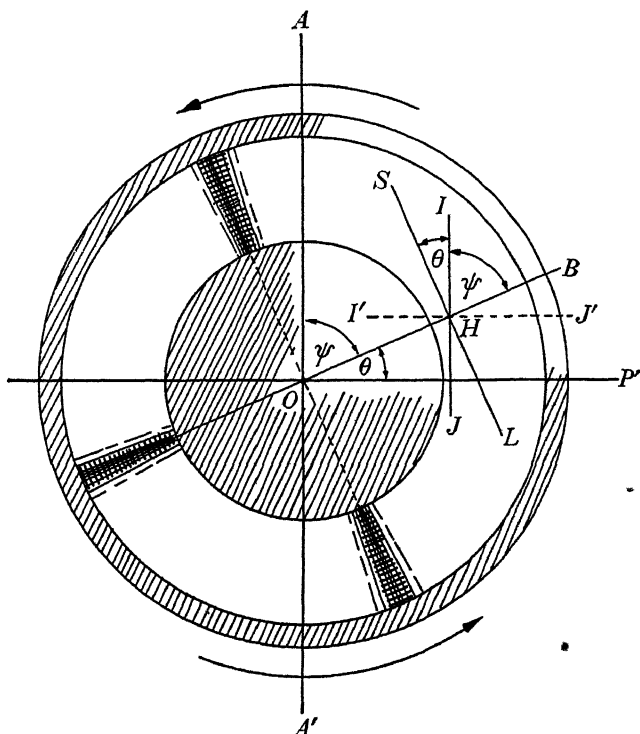


FIG. 2. The black cross of isocline. If AA' and PP' are the planes of polarisation of the optical system, then for OB to be a dark brush, a particle with its centre at H must have its optic axes in the directions IJ and $I'J'$, parallel to AA' and PP' . SL , the perpendicular to the radius OB , is the direction of the stream lines. The geometry of Ψ and θ is then fixed as shown.

tension perpendicular to each other and inclined at 45° to the stream lines. The measured angle of isocline should always be, therefore, 45° , and this has been experimentally confirmed. Such photoelastic birefringence in transparent plastic models has been used to study strains in engineering structures (Coker and Filon, 1931). (b) *Form-birefringence* (Wiener, 1912) which arises from the orientation of optically isotropic rod-shaped particles, the size of which is small compared with the wave length of light. Such a system in flow shows birefringence provided that the refractive index of the particles is different

from that of the dispersion medium. (c) *Intrinsic birefringence*, which is due to orientation of optically anisotropic particles. Form-birefringence disappears when the refractive index of solvent and solute are identical. For example, measurements (at rest) have shown that this does not occur, either with gelatin (Ambrohn, 1915) or with myosin (Stubel, 1923; Weber, 1933, 1934). Hence the particles must be optically anisotropic.

To correlate viscosity with particle shape, according to the theoretical treatment outlined above, it is necessary to deduce a distribution function for the position of the particles with respect to the stream lines. The expression of Boeder (1932) cannot be integrated and he gave graphical solutions in the form of curves. Robinson (1939) made the very important advance of using his experimental flow-birefringence data as a measure of the distribution of the particles and, hence, of their contribution to the dissipation of energy by precessional motion. He arrives at the expression:

$$\eta/\eta_0 = 1 + \frac{1}{2}Vf^2 \left[1 - \frac{\Delta}{\Delta_{max.}} (1 - \cos^2 \psi) \right]$$

where Δ and $\Delta_{max.}$ are the observed and maximum intensities of birefringence, f being the ratio of particle length to particle radius. If we neglect the term in square brackets and place it at unity, we get Kuhn's equation less the arbitrarily added Einstein 2.5 V term. If we evaluate the term in square brackets in Robinson's equation, we find that it can vary from 2 to zero.

The approximate form $\eta/\eta_0 = 1 + \frac{Vf^2}{4}$ can therefore be used for an estimate of f .

We may now sum up the position. Three ranges of particle size must be recognised: (a) Very long thin particles which are nearly completely oriented by moderate rates of shear. These are rather rare, e.g. "aged" sols of V_2O_5 (Freundlich, Stapelfeld, and Zocher, 1924; Freundlich and Schalek, 1924). (b) Intermediate sized long thin particles whose behaviour can be studied over the whole range of ψ from 45° to 90° . TMD virus (Robinson, 1939) and mercuric sulphosalicylic acid (Hatschek, 1928) are good examples. (c) Small elongated particles the behaviour of which in the viscosimeter never passes random orientation. This is the largest class, comprising many polymers, although some of these have been found to show flow-birefringence, e.g. methyl methacrylate (de Rosset, 1941), polystyrenes (Signer and Gross, 1933; Signer, 1936).

All that has been said above assumes that only one parameter changes, e.g. axial ratio of the particle. When, however, we meet with other changes, such as the degree of dispersion of the sol, aggregation and disaggregation, changes of intermicellar forces, etc., the situation becomes very much more complicated, and a full analysis of any given case is as yet not always possible. A

beginning has been made by considering the effects of polydispersity (Sadron, 1937, 1938; Sadron and Mosimann, 1938). The effects of electrical fields in counteracting orientation due to shear (Björnsthål, 1935; Björnsthål and Snellman, 1937) may be of use in analysing the more complicated cases.

As for the adhesions between particles which intermicellar forces bring about, we know that something of the kind exists in sols of the TMD virus, because hexagonal packing occurs in the plane at right angles to the length of the particles, if the concentration is high. For dilute sols the forces concerned must always be small, and sound hydrodynamical treatment may ignore them. Adhesion forces act in two ways. Weak attachments will be broken by moderate shearing, and will show themselves only as a high initial anomaly or very small yield-point, if the solution is sufficiently concentrated for a continuous meshwork to be built up on standing. Alternatively, adhesion forces may act by building up micelles or bundles of particles which cannot be broken by moderate shearing. Such bundles need not bear any geometrical similarity to the constituent particles. Thus a bundle of fibrils may become, by lateral aggregation, as broad as it is long, and so approximate to a spherical particle. On the other hand, there may be any intermediate degree of elongation, ending in the unlikely case of end-to-end packing, but including sliding parallel extension and closure.

Coming now to the type of the instrument used, it is important to note that the coaxial viscosimeter (Couette, 1890) is essential for a number of reasons. (a) The variation of rate of shear is very small across the annulus, as compared with that across the capillary in the Ostwald viscosimeter. Where viscous anomaly is present, this is a serious matter, for the liquid will be subject to a much higher rate of shear near the walls of the capillary than near its centre; calculations of mean rates of shear under these conditions have no physical meaning. (b) Steady equilibrium flow conditions are reached in the coaxial, but not in the capillary, viscosimeter. (c) Optical observations in the coaxial cell will indicate when random orientation equations no longer apply, and furnish important alternative information about the status of the particles. (d) Observations in the coaxial cell can be continuous so that changes in the rheological and other physical properties can be followed moment by moment, and compared, if desired, with simultaneously proceeding chemical changes.

The value of the Couette type of viscosimeter was not overlooked by earlier workers on the physical chemistry of the proteins, especially those who studied flow-birefringence in the coaxial cell, but they reported a lack of success in using it as a viscosimeter (von Muralt and Edsall, 1930*a*, pp. 339, 363; 1930*b*, p. 852). We believe that their difficulties were due to lack of appreciation of the important part played by the surface film of protein solutions in the Couette viscosimeter as normally employed (see below, p. 219).

Definitions and Units

The intensity of flow-birefringence in a protein sol subjected to shear stress is a function of the number of anisometric particles present (protein concentration), the degree of their own optical anisotropy, the perfection of their orientation (the resultant, for a given axial ratio, of the opposing forces of shearing stress and thermal disorientation), the depth of the solution through which the light beam passes, and the other conditions governing particle shape and size such as pH and salt concentration. We express it here in terms of Δ° : the angle through which the analysing Nicol must be rotated to extinguish the plane-polarised light emerging from a $\frac{1}{4}$ -wave plate.² The double refraction itself can be obtained at once from this figure by means of the following relations:

$\frac{\Delta^\circ}{180^\circ} = \Delta p$ the phase-difference in wave lengths between the two components of the elliptically polarised light; and $\frac{\Delta p \cdot \lambda}{S} = N_e - N_o$ the double refraction (the difference between the two refractive indices, N_e for the extraordinary and N_o for the ordinary beam), where λ is the wave length of the light source, and S the depth of solution under examination, expressed in the same units (Ambronn and Frey, 1926).

The angle of isocline, ψ , is by definition the larger of the two angles which the cross of isocline makes with the crossed planes of polarisation of the polarising and analysing Nicols. It is here interpreted as a measure of the degree of perfection of the orientation of the anisometric particles in the stream lines under shear stress. It varies between 45° for nil orientation and 90° for perfect orientation.

The relative viscosity, η/η_0 , is the ratio between the mirror-deflection responses of the suspended central cylinder to the torques exerted by the solution under examination, and by distilled water or equivalent electrolyte solution, respectively, at the same temperature.

Anomalous viscosity is the departure of relative viscosity from independence of the shear rate. Unfortunately no satisfactory means has yet been devised for its quantitative expression.

² Monochromatic plane-polarised light passing through a birefringent medium is transformed into elliptically polarised light. Now the planes of vibration of the two components (fast and slow) of the light passing out of the streaming sol are inclined at a certain angle to the original plane of polarisation of the light from the lower Nicol. The Bravais $\frac{1}{4}$ -wave plate, used as a Senarmont compensator, by virtue of its own birefringence, converts the elliptically polarised light back into approximately plane-polarised light. The analyser therefore has to be rotated through a certain angle (Δ°), proportional to the phase difference of the two components of the elliptically polarised light, to give approximate extinction, *i.e.* to allow the cross of isocline to manifest itself at a new position, and this occurs at 45° to the former.

Technique

For the physical measurements in the present work we used two pieces of apparatus: (a) a small cell placed on the stage of a polarising microscope, and (b) a Couette viscosimeter arranged for simultaneous optical determinations.

The Microscope Cell.—With the cell on the microscope stage, measurements of flow-birefringence intensity and angle of isocline are readily made. The cell is of glass, fitted into a brass tube forming part of a pulley and ball race; the bottom is also of glass. The central stationary pillar is a glass rod, held in position by three struts from a brass cover attached to the base by three bolts and nuts. The dimensions of the cell, which holds 0.85 ml. when full, are given in Table I for comparison with other apparatus. Illumination is provided by a G.E.C. Osira laboratory type monochromatic sodium lamp, giving pure light of wave length $590\text{ }\mu$ and the outer wall of the cell can be rotated at speeds up to 600 R.P.M. by a geared electric motor with suitable rheostats, using a rubber belt.

The Swift-Dick fixed-stage polarising microscope was provided with a $\frac{1}{4}$ -wave plate between the ocular and the analysing Nicol. Owing to the depth of the annular space in the cell, above the polarising Nicol and stage lens, it was necessary to remove the ocular normally present and insert a telescopic tube, mounting objective and ocular, of some 3 inch focal length. This raised the height of the whole microscope, but the analysing Nicol holder and the $\frac{1}{4}$ -wave plate easily fitted on to the top of the telescopic tube. The $\frac{1}{4}$ -wave plate was kept permanently in position, its accuracy of setting being checked from time to time. The flow-birefringence intensity could then immediately be found by noting the angle through which the analyser had to be rotated in order to obtain the narrowest and darkest cross at 45° to the former cross of isocline.

The angle of isocline was obtained by placing a pointer just above the annular meniscus at 90° to the polariser. Then, with the analyser crossed to give extinction, the coupled Nicols are turned together through the longest possible range till one of the brushes of the cross of isocline centres on the pointer. This angle is the angle of isocline.

Comparison of Shear Rates.—The shear rate of a coaxial cell is its most important characteristic. It is derived from the formula

$$2\Omega \frac{R_1^2 R_2^2}{r^2(R_2^2 - R_1^2)}$$

where Ω is the speed of revolution in radians per second; R_1 the radius of the inner, and R_2 that of the outer, cylinder; and r the radius of a circle half-way across the annular space. Values which show how our apparatus compared with other instruments will be found in Table I.

The Coaxial Viscosimeter.—The coaxial viscosimeter is a smaller form of the instrument designed by one of us and used by Robinson (1939). As no description has yet been published, some details are given here. It is one of the Couette type but with the essential new feature that simultaneous measurements may be made of flow-birefringence and viscosity. The two properties are therefore measured under formally identical flow conditions (see below), a requirement persistently ignored by most workers in this field, who have measured one property in the coaxial cell and

the other in the Ostwald capillary viscosimeter in which the flow conditions are quite different and may not reach hydrodynamical equilibrium.

The design aims at maximum steadiness of flow combined with ease of dismantling and reassembly without loss of precision (see Fig. 3). It consists of four units, each

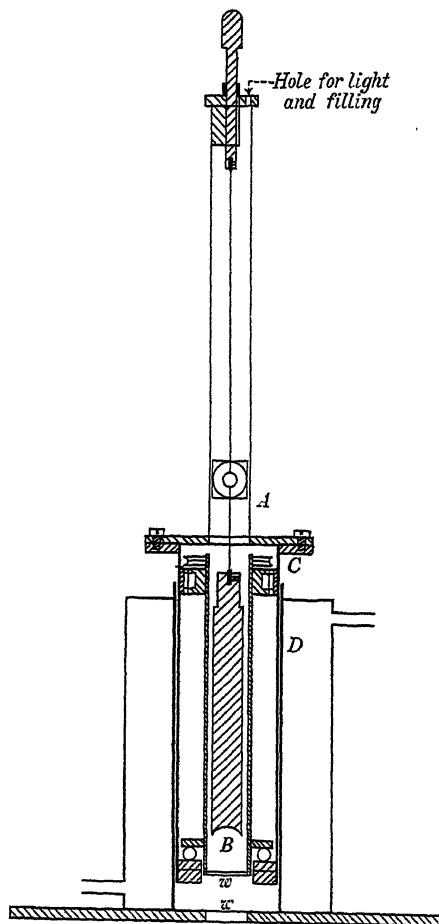


FIG. 3. Construction of viscosimeter.

of which is constructed and trued up on the lathe before assembly into the instrument, the deviation of which from a truly coaxial set-up is negligible. The units consist of: *A*, inner hanging cylinder (see Taylor, 1936), torsion head and its supporting tube mounted on the top plate of the instrument with or without a space permitting access at this point: *B*, rotating cylinder, the top of which is fixed inside a roller bearing, while near to the bottom it carries the upper plate of a ball-bearing. After removal of *A*, *B* can be lifted out. *C* is the supporting cylinder for *B*, which makes a tight

sliding fit in it (shown in the diagram as a finite clearance): D is the water jacket and base of the instrument. The correct position of C in D is fixed by a small set-screw (not shown in the diagram). The suspension wire is soldered into very small chucks which slip into the ends of the hanging cylinder and the torsion head where they are fixed by set-screws. The clearance between the bottoms of the two cylinders is adjustable by moving the rod in the torsion head and its magnitude can be determined by lowering this rod until the suspension wire sags and then raising the rod to any desired level. The base of the instrument and the bottom of the rotating cylinder are provided with glass windows (w) through which the polarised light passes upwards and emerges through a cut away space in the torsion head block. No glass window was fitted here since this space is useful as access to the annulus for removing bubbles, filling, and emptying, etc. By using a thin glass tube with a fiducial mark to coincide with the top of the torsion head, the liquid in the annulus can be adjusted to any level required. The base plate is provided with levelling screws.

The dimensions of the instrument are a compromise between the theoretically best and limitations imposed by the small amounts of liquid available. To allow for end-correction at the bottom of the cylinders, the clearance needs to be large compared with the width of the annulus. The hemispherical form of the bottom of the hanging cylinder is provided to enclose an air bubble and so further reduce end-correction (*cf.* Mallock, 1888). The length of the annulus needs to be considerable to get sufficient torque on the inner cylinder at low rates of shear. The result is that R_1 and R_2 are rather small but the clearance between them is thin enough to ensure a small variation of the rate of shear across the annulus. There is a practical lower limit to the width of the annulus below which sufficient illumination of the field is not obtained for the observation of the flow-birefringence. The diameters of the two cylinders and other measurements, with the calculated shear rates, are given in Table I. In the original instrument, the two cylinders were made of stainless steel but chromium-plated brass was used successfully later. We did not meet with spurious birefringence arising from reflections off these surfaces (*cf.* Frey-Wyssling and Weber, 1941), partly because our light beam was very nearly parallel, and partly because protein solutions are so highly light-absorbing. We have not thought it worth while to introduce any correction for this, since the main function of our data is in relative comparisons.

In use, the instrument's outer cylinder can be evenly rotated at speeds varying from 0.3 to 200 R.P.M., while the lagged jacket allows of accurate temperature control. This was carried out by letting water at known temperature flow through the apparatus; it comes in a lagged tube a short distance from a large volume Warburg manometer tank electrically heated and controlled by a thermostat and relay. Most of the experiments here reported were made at 20°C. The roller bearings and ball race of the rotating outer cylinder (the space between B and C in Fig. 1) operate in medicinal paraffin oil, and this lubricating medium also fills the whole of the inner jacket space, including that which intervenes between the glass bottom of the instrument and the glass bottom of the rotating cylinder. It is, of course, essential that the bearings should be absolutely free from rust or other particles, and from time to time, even when no visible particles can be detected, it is necessary to remove the rotating cylinder and bearings, soak them in benzene, and polish the races with a small amount of mild abrasive such as brasso.

The drive for the rotating cylinder is a matter of much importance. We obtained excellent results by using a $\frac{1}{2}$ -h.p. electric motor and a stand of adjustable pulleys

TABLE I
*Comparison of Properties of Coaxial Cells**

Cell	Type	Inner cylinder used	R_1	R_2	r	Width of annulus	For 100 R.P.M. Ω	Shear rate	Height of column of liquid sheared
			cm.	cm.	cm.	cm.	radians/sec.	cm./cm./sec.	cm.
von Muralt and Edsall (1930)	Optical only	I	0.91	2.0	1.45	1.09	10.5	10.4	9.9
		II	1.30	2.0	1.65	0.70	10.5	22.6	9.9
		III	1.80	2.0	1.90	0.20	10.5	98.0	9.9
Böhm and Signer (1931)	Optical only	—	2.30	2.495	2.40	0.195	10.5	131.0	4.7
Sadron (1936)	Optical only	I	2.42	2.55	2.485	0.13	10.5	199.0	9.8
		II	2.50	2.55	2.525	0.05	10.5	445.0	
		III	2.53	2.55	2.54	0.02	10.5	1360.0	
Robinson (1939)	Optical and viscosimetric	—	1.73	2.86	2.30	1.135	10.5	18.8	20.7
Present work: Microscope cell	Optical only	I	0.15	0.295	0.222	0.145	10.5	13.1	At first
		II	0.245	0.295	0.270	0.05	10.5	55.9	1.5
									Later lengthened to 3.0
Couette	Optical and viscosimetric	I	0.40	0.785	0.593	0.192	10.5	7.7	At flood level
		II	0.64	0.785	0.713	0.072	10.5	44.0	10.0
									At low level 8.0

* Cf. Edsall, 1942, p. 275.

similar to those used for Warburg manometer shakers. From the motor to the final pulley in the stand the connections were round-section leather belts, from the final pulley to the drive-wheel (which at every revolution was made to sound a bell) the connection was stout string, and from the drive-wheel to the apparatus itself, the connection was a length of cotton seven times the distance between the wheels and yet knotted only in one place. Suitable pulleys for adjusting the passage of the cotton-

drive were found (in the absence of small pulleys on ball bearings) in the pulleys used for the drives of dentists' drills; these are excellently machined, and one is provided on a ratchet mechanism as an idler for giving the exact degree of tautness required. If such a cotton belt is kept free from oil by an occasional cleaning with ether, it gives (as no other type of drive in our experience did) smooth enough running for acceptable readings on the galvanometer scale.

The mirror on the torsion wire, the lamp illuminating it, and the scale on which the image shone, were of normal galvanometer pattern. The torsion wire itself was at first of steel, but later on, when, as will be described below, it became essential for the hanging cylinder to be completely immersed in the solution under examination, the wire broke through corrosion too frequently and phosphor bronze was used instead, with excellently reproducible results. It is necessary to calibrate the behaviour of every wire with pure distilled water; with steel wires we sometimes, though by no means always, found a double instead of a single linear relation between speed of rotation (shear force) and deflection (response to torque). Instead of a single line from the origin at a certain angle, throughout the range, the relation would follow such a single line until a certain speed was reached, and thenceforward follow another single line at a lesser angle with the abscissa. This, however, did not occur till fairly high speeds were reached, was not readily detectable with the experimental solutions themselves, and has not been observed with phosphor bronze wires.

For varying the speed it was found convenient to have a rheostat conveniently placed for the observer at the galvanometer scale, so that the result for any speed desired could be read from rest up to 2.4 R.P.M., then from 2.4 R.P.M. up to 6.0 R.P.M., from 6.0 to 15 R.P.M. and so on, the belting and pulleys being changed only at these intervals. It was also found convenient to suspend the galvanometer scale from a runway made of curtain railing thus permitting some lateral movement, but with a locking device to keep it at any desired place. Unlike galvanometer technique, the constant manipulations of the cylinders affect the position of the mirror on the suspension wire, and its exact angle relative to the light beam cannot be easily controlled.

The optical equipment used for measuring the flow-birefringence of solutions in the viscosimeter is shown diagrammatically in Fig. 4. The light source is a 3000 watt B.T.H. projector lamp, enclosed in a box of asbestos and tin, and cooled by a powerful electric fan. This illumination is the minimal adequate, and an even stronger light source would be desirable, owing to the great scattering of light by protein solutions. The light is conducted horizontally through a black tube (since such lamps must burn vertically), concentrated slightly by a biconvex lens, and reflected up into the instrument by a plane mirror, the 45° setting of which can be exactly adjusted. This part of the apparatus is contained within a small but stout wooden table which supports the instrument above, while below it rests upon a length of tree trunk about 1 foot 6 inches high. Pads of Sorbo rubber intervene between the table and the base, and between the base and the floor; these insulate the whole structure fairly effectively from stray vibrations in the building. Above the plane mirror is a shelf on which rest two pyrex Corning filters (Nos. 978 and 349), giving a band of wave lengths centering on the sodium line (550-630). The light next passes into a large polarising Nicol, taken from a mineralogical lecture room type lantern; the carriage of this is capable of wide rotation and provided with a handle and pointer to a scale just below the

viscosimeter. Just above the lenses of the polariser there is a small planoconvex lens. By this means a powerful beam of polarised light ascends over nearly the whole area of the annulus, but the viscosimeter is placed slightly excentrically in order to obtain

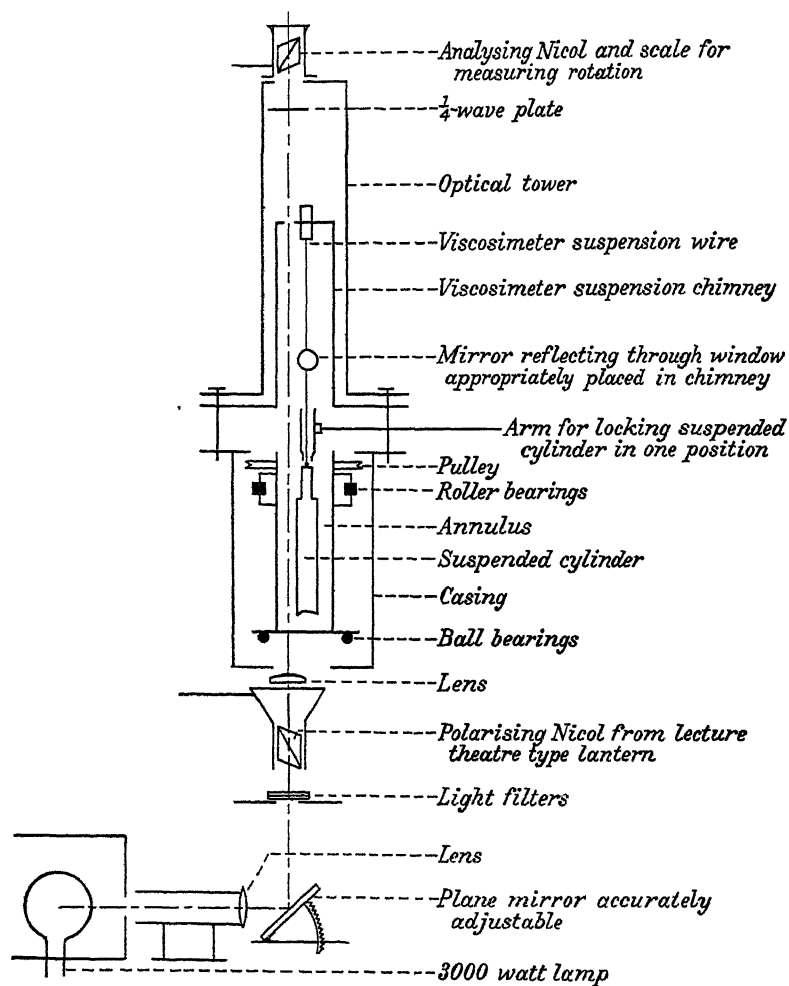


FIG. 4. Diagram of optical arrangements in the viscosimeter.

more perfect illumination of one half its disc. The light ascending in the viscosimeter's annulus is not perfectly parallel, but very nearly so. At the meniscus it passes out into the chimney of the suspension, at the top of which there is a window so arranged as to enable the observer to see a good deal more than half of the whole disc and annulus, and hence three of the four brushes of the cross of isocline. Outside the brass suspension wire chimney, and attached like it by the same brass bolts to the lower parts of the instrument, there is another chimney or tower made of stout

cardboard tubing, and bearing fitted to its upper end the $\frac{1}{4}$ -wave plate and the analysing Nicol with its scale of measurement of rotation.

One further component of the apparatus remains to be described. It is usually necessary, when measuring flow-birefringence, to employ a higher speed of rotation than when measuring anomalous viscosity; it is therefore necessary to have some braking mechanism whereby the central cylinder can be maintained in a stationary position. It may be said at this point that only in the case of the TMD virus, in the work of Robinson (1939), has it ever proved possible to measure the flow-birefringence and viscosity of a protein strictly simultaneously. No other protein so far studied, even myosin, has sufficient specific flow-birefringence intensity. At the same time, it is obviously a great advantage to take the optical and viscosimetric measurements upon the same sample of protein, at unchanged concentration and temperature, and in the presence of the same added substances, etc. The suspension wire of the hanging cylinder, therefore, passes through a short glass tube, as shown in the diagram, the lower surface of which is ground. This tube is secured in a brass holder electroplated with silver, which in turn is attached to a rod passing horizontally out of the apparatus, and capable of being carefully raised and lowered by an adjacent rack and pinion mechanism. When it is desired to fix the hanging cylinder in one position, this glass tube is lowered so that it holds the cylinder tight with an annular but regular pressure; the shear force can then be increased as much as is necessary to obtain good flow-birefringence readings without any danger of straining the suspension wire.

Degree of Accuracy Obtainable in Readings.—The brushes of the cross of isocline, under good conditions, are quite sharp. In the microscope cell the accuracy obtainable in the readings was $\pm 2^\circ$ over the major part of the range but towards the ends (below 10° and above 100°) the experimental error was somewhat higher. In the viscosimeter the accuracy obtainable in the readings was rather less, $\pm 5^\circ$ over the major part of the range.

Comparison of Readings in Microscope Cell and Viscosimeter.—Convenient speeds of rotation are some 8 times higher in the microscope cell than in the viscosimeter (300 to 500 R.P.M. as against 50 R.P.M.), but the depth of solution is some 3.3 times less (1.5 to 3.0 cm. as against 8 to 10 cm.). The shear rate in the viscosimeter as generally used (cylinder II) is 3.36 times that in the microscope cell as generally used (cylinder I). Hence the flow-birefringence readings in the two instruments on the same myosin sample are not very far apart; it is necessary, however, to use more dilute sols in the viscosimeter when viscosimetric readings are simultaneously undertaken.

General Types of Effect Observed

The general types of effect observed follow from the theoretical introduction given above. Fig. 5 reduces the facts to their simplest form (*cf.* Lawrence, 1937). In Fig. 5 *a* and 5 *b* the deflection of the mirror, δ , on the suspension wire responding to the torque to which the hanging cylinder is subjected, is plotted against the shear force applied to it, which is proportional to the speed of rotation of the outer cylinder in revolutions per minute. The lines marked *W* represent the deflections given by water alone at the experimental temperature, or by whatever salt solutions are used as controls for the dilute protein

sols. Line *A* represents that given by proteins with approximately spherical molecules or particles. The flow here is normal or Newtonian, and the relative viscosity (shown as *A*₁ on the accompanying diagram, Fig. 5 *c*) is therefore constant whatever the shear force. Proteins with anisometric molecules, particles, or aggregates, however, give lines such as that shown by line *B* in

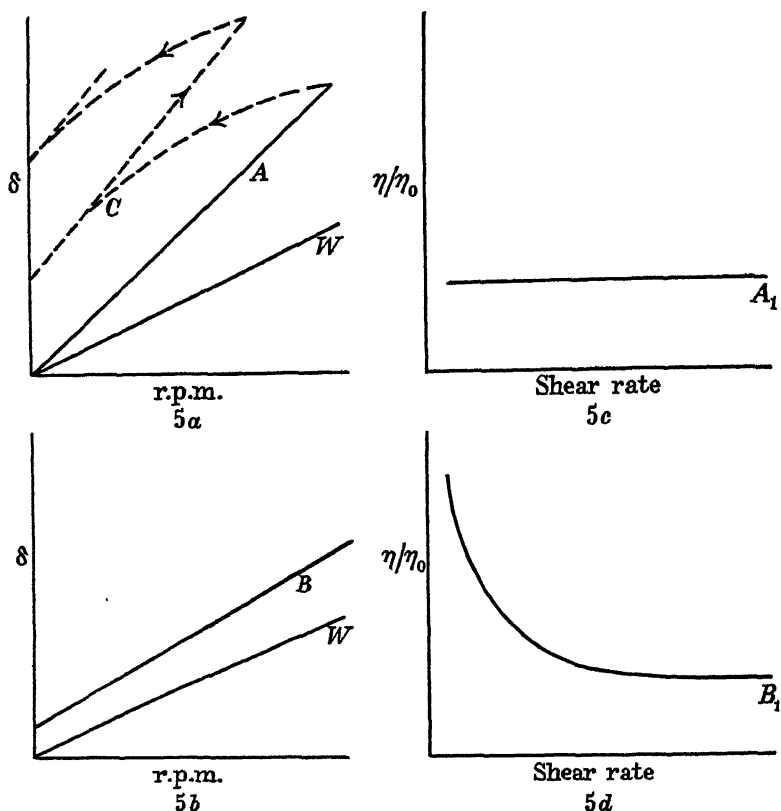


FIG. 5. General types of effects observed.

Fig. 5 *b*, the readings being abnormally high at low shear rates and falling off as high rates are approached. Such *anomalous flow* means that the relative viscosity varies with the rate of shear (as in *B*₁ on the accompanying diagram, Fig. 5 *d*), falling from a very high value to a constant or approximately constant level when perfect orientation of the long particles in the stream lines is attained. These two cases are the simplest met with, as will be seen from the references to specific proteins in the following paper.

Other proteins exhibit more complex behaviour. Thus a series of protein deflection readings may first follow a line similar to *A* in Fig. 5 *a*, but when slow

speeds are again applied, an "anomalous return" is obtained, cutting the ordinate at a higher level. The cycle can then be repeated, with a return to the ordinate (rest) at a higher level still. It should follow then that by continuous rotation of the outer cylinder at constant speed, the deflection should continuously increase instead of remaining constant, and this is accordingly found to happen (see below). After a definite time this "stretching" process comes to an end, and no further rise in deflection is observable. We undoubtedly have to deal here with the gradual formation of a monolayer or multilayer on the air-water interface of the solution. This will be fully discussed in what follows (p. 220).

We have not so far found any protein which showed anomalous flow initially, followed by anomalous return and polyfilm formation; the probable significance of this fact will become clear later.

It is of interest that something like our "anomalous return" was observed by Hatschek (1913) in gelatin sols and described by him as a "hysteresis" effect. Later Ostwald (1927) came near to what we believe to be the correct explanation, though he did not actually give it, in his suggestion that the effect was due to film formation at the surface of the inner cylinder.

In the irreversibility of the phenomenon and its non-repeatability on the same sample of protein, this "stretching effect" is reminiscent of the compression of protein films by piston oil in the experiments of Li and Wu (1932) extended by Langmuir and Waugh (1940). A compressed film gradually contracts because weakly hydrophobe groups are forced into solution, then later, when released, cannot expand again to its original area; and several cycles of this kind may be followed. In our case the shearing rotation of the surface film seems to invite more and more protein molecules into it, perhaps because they are better packed therein than normally. Eventually the maximal surface area is covered with a maximally thick film.

The coaxial viscosimeter is, of course, not indispensable for the detection of anomalous flow, since solutions may be forced through capillary viscosimeters under different pressures or suctions and hence at different average shear rates. The capillary method suffers indeed from severe theoretical deficiencies, as described above. But it is important to note that the capillary method cannot measure the changing relative viscosity from moment to moment, and without the coaxial viscosimeter it would therefore have been impossible to examine the "stretching" effect just described, and hence to make a separate analysis of film and bulk viscosity.

More remarkable is the following phenomenon. We have frequently observed that if, after the "stretching" process has proceeded a short while or has come to a conclusion, the protein solution be removed from the viscosimeter and filtered, upon being put back it will give a picture like that of Fig. 5 *b*, curve *B*; *i.e.*, anomalous flow. It is extremely probable that this is due to

the spinning off of anisometric protein particles from the surface film, or to their liberation from it once having been formed there, when the solution is removed from the apparatus. This may possibly throw some light on how anisometric molecules are formed in the living cell.

A word may be added here about the assessment of anomaly. During the actual observations we found it impossible to predict how the diagram would appear, and it is usual to obtain either marked anomaly on the graph or definitely normal flow, but there is a class of cases where the line describing the set of deflection points cuts the ordinate so near the origin that its status cannot readily be determined. We have not chosen any of these uncertain cases for discussion in this paper.

It should also be remarked that in the following papers in the series, when comparing the relative viscosities of solutions showing anomalous flow, we always chose shear rates by which the relative viscosity had sunk to its minimal and constant plateau.

Film and Bulk Viscosity of Protein Solutions

The property which most proteins have of forming monolayers and multilayers at the air-water surface is well known, and any attempt to study protein solutions in the coaxial apparatus must necessarily reckon with it. The "stretching" phenomenon just described, *i.e.* the continuous rise in deflection when the solution is rotated in the annulus at constant speed, until a final value is reached, was soon found to depend upon the presence of the surface film. If the inner cylinder, instead of being partially immersed (as has nearly always been the practice in coaxial viscosimeters hitherto)—see Fig. 6 *A*—is entirely immersed, so that the suspension wire alone passes through the surface film—see Fig. 6 *B*—the "stretching" effect is never observed.

This is illustrated in Fig. 7. Curve *a* shows the behaviour of a 0.10 per cent solution of freshly dialysed crystalline ovalbumin, rotated continuously at 11.9 R.P.M. at low level. After about 15 minutes the deflection had reached a maximum and, as usual, remained there. Curve *b* shows the same solution at flood level. No "stretching" took place, until at the point marked with the arrow sufficient of the solution was removed to bring it down to low level, after which there followed on further rotation precisely the same effect as before. It is probable therefore that not only is a solid multilayer formed, but also, since the surface area of the meniscus must, owing to centrifugal force, be very slightly larger during the rotation than at rest, the multilayer buckles when the rotation ceases. In any case it is clear that the torque on the wire alone is quite insufficient to manifest the properties of the surface film.

These facts suggest that at the low level position, we have to deal with surface or film viscosity, while at the flood level position we have to deal with bulk viscosity. This is further demonstrated by the following experiments.

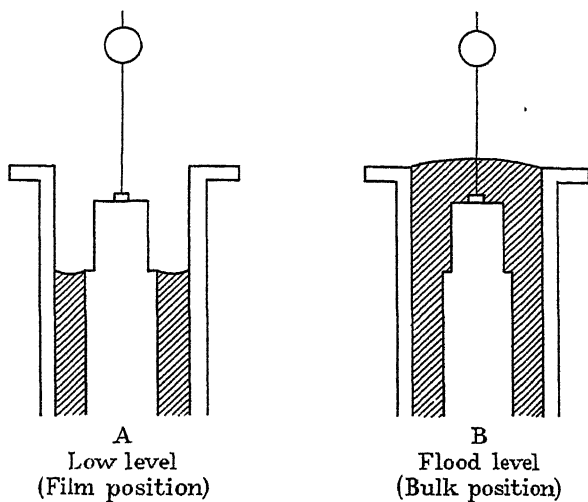


FIG. 6. Conditions in viscosimeter for film and bulk viscosity measurements.

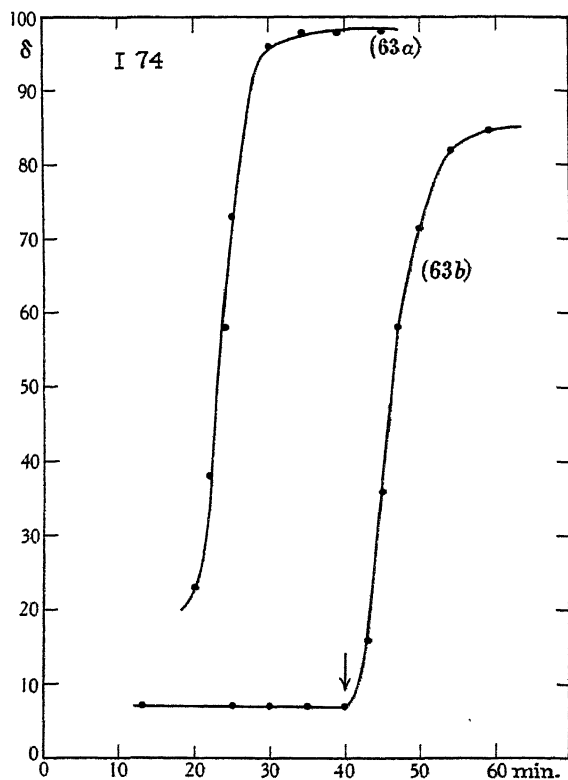


FIG. 7. "Stretching" effect of ovalbumin surface film; building of multilayer.

In the first place we may keep the film and the shear rate constant while varying the bulk torque. To do this we must immerse the same suspended

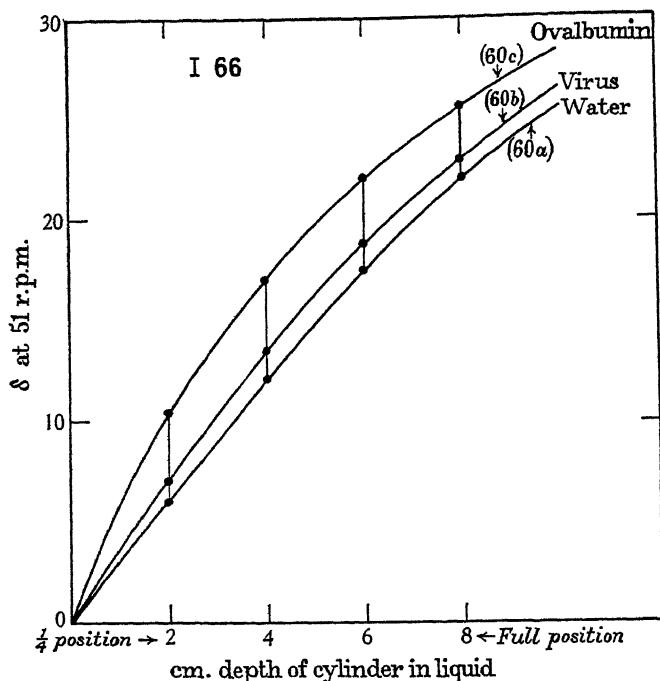


FIG. 8. Variation in bulk torque with film and shear rate constant.

TABLE II

Deflections of Inner Cylinder Mirror under Varying Bulk Torque by Virus Nucleoprotein and Ovalbumin

Cm. of cylinder I immersed in solution	Solution	Deflections above water value	
		TMD virus	Ovalbumin
	ml.	mm.	mm.
8 (normal low level)	13.39	2.0	7.0
6	10.55	2.5	9.0
4	7.66	3.0	9.5
2	4.81	2.5	8.5

cylinder in different amounts of solution. The results of one of several such experiments are shown in Fig. 8. The deflections observed while running at 51 R.P.M. and with the cylinder immersed to different extents, are plotted for distilled water, 0.025 per cent TMD virus, and 0.03 per cent crystalline ovalbumin, all at 20°C. As can be seen from the plot and from the figures in Table II

the difference between the water values and those of the two protein samples is substantially the same to whatever depth the cylinder is covered, yet the bulk phase and hence its torque is progressively less in amount. Had the bulk phase been the main determining factor, the difference would have been expected to show a regular decrease. One may therefore conclude that although the bulk torque of the water alone does of course decrease as the cylinder is less and less immersed, the torque of the protein viscosity is effected entirely through its surface film. In order to measure bulk phase protein viscosity, therefore, conditions must be found in which the surface film does not play an important part. Flood level provides such conditions.

The second experiment is to keep the film and the bulk torque constant but to vary the shear rate. To do this we must use suspended cylinders of different diameters—in this case, cylinders I and II, of 0.8 and 1.2 cm. diameter respectively, in each case at flood level. If the surface film were here responsible, or intervening to any extent, agreement between the viscosity values would not occur. The line plotted through the deflections read with cylinder II for a protein solution might not fall very much or even at all when cylinder I was used, and would certainly not fall to exactly the correct place. Results obtained in two typical experiments are shown in Fig. 9 for a protein showing normal flow (0.1 per cent crystalline ovalbumin) and in Fig. 10 for a protein showing anomalous flow (0.078 per cent flow-birefringent rabbit myosin). The figures in Table III indicate that the relative viscosity at any rotation speed appears substantially the same whichever cylinder is used. It is interesting to notice that the results with the small cylinder are consistently slightly lower than those with the large cylinder, whereas if the surface film had been playing any part, the error would have been in the opposite direction. It may therefore be concluded that the surface film plays absolutely no part when the flood level position of the suspended cylinder is used; *i.e.*, that the torque on the suspension wire is quite negligible as compared with the bulk torque under these conditions.

In Fig. 10 the curves of relative viscosity have been inserted. It is interesting to notice how the greater shear rate of the larger cylinder with its narrower annulus orients the particles in the stream lines about 2 R.P.M. earlier than that of the smaller cylinder, with its broader annulus.

One may therefore assume with fair confidence that under low level conditions we have to deal with the flow of fibrillar or plate-like particles within the surface film. The presence of such films was often verified, by noting the behaviour of talc motes scattered on the surface of the solution in the apparatus. Conversely, under flood level conditions we have to deal with the flow of particles within the bulk phase. In conformity with this, the whole of our experience has shown that at flood level all protein solutions have to be much stronger than at low level; *e.g.*, of the order of 0.1 per cent protein as against

0.001 per cent protein. The multilayers formed at the stronger concentrations are too tough and rigid for the range of the instrument.

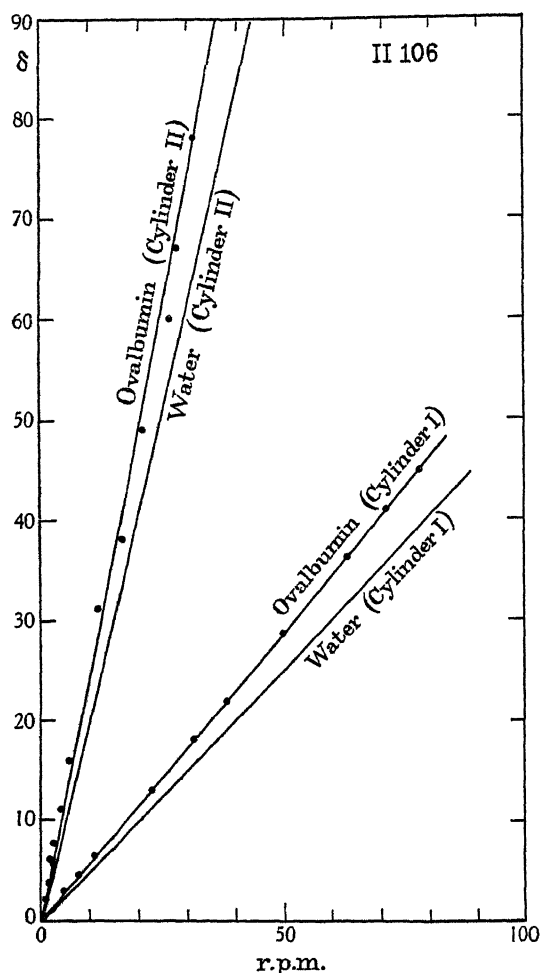


FIG. 9. Variation in shear rate with film and bulk torque constant (a) flow diagram for ovalbumin.

It may be added that there are theoretical grounds for believing in the correctness of the above conclusions. Surface viscosities of protein solutions vary over a wide range, but a value of 50 surface poises could reasonably be taken for ovalbumin solutions as used here. In this case, it may be calculated that the surface film has a viscosity 300 times as great as that of the bulk phase. Experiments of an analogous kind to those described above have been done

by Crisp (1942) who determined the change of log decrement with depth of immersion of a surface viscosimeter of the swinging vane type. Here again the surface viscosity was of the order of more than 100 times that of the bulk.

Calculations moreover may be made comparing the radii of suspension wire and cylinder (in this case 0.003 and 0.40 cm. respectively). For a given

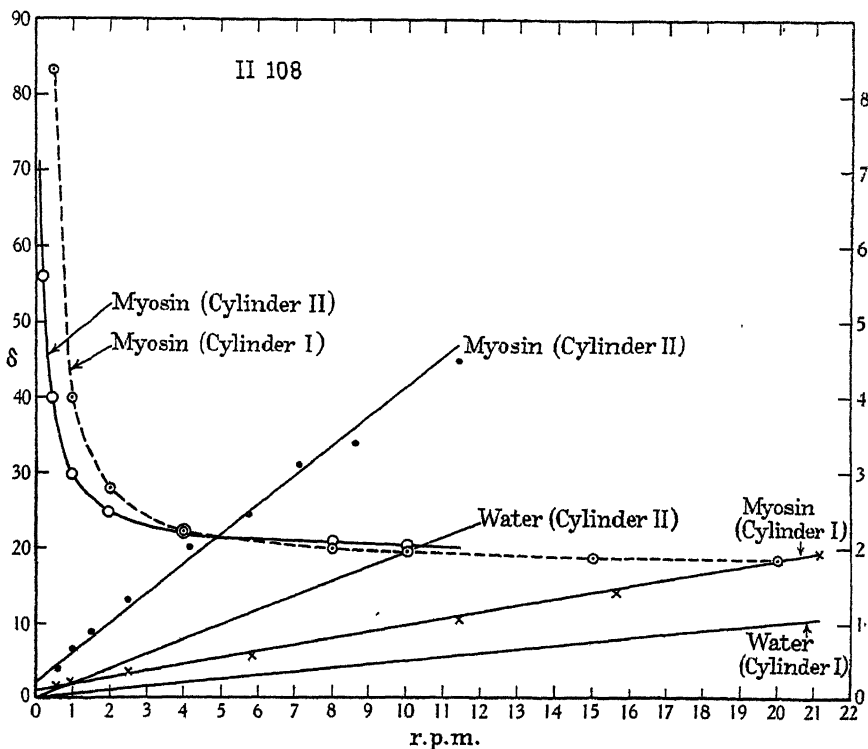


FIG. 10. Variation in shear rate with film and bulk torque constant (b) flow diagram for myosin.

protein solution, the torque on the cylinder would be 13,900 times that on the wire.

It is not easy to understand how the particles move with respect to one another in orienting to shear stress within an apparently relatively solid surface film, but as may be seen from Fig. 11, the relative viscosity of such films falls with increasing shear rate in a slow and regular way, considerably slower, indeed, than in the case of bulk measurements. If the film were to break away along the edge of its attachment to one or the other of the coaxial cyl-

inders, the initial torque on the hanging cylinder would be very high, but the deflection would instantly return to zero, or rather, to the bulk level for water as soon as the breakage occurred, and no slowly falling curve would appear. If the film were to break up into isolated flocs, the apparent relative viscosity

TABLE IIIa

Relative Viscosities of Myosin and Ovalbumin at Varying Shear Rates; and Ratio of the Torques

	Speed of rotation	Relative viscosity	
		Small cylinder (I)	Large cylinder (II)
Ovalbumin	R.P.M.		
	10	1.21	1.26
	20	1.17	1.22
	30	1.16	1.20
Myosin	40	1.14	1.18
	4	2.25	2.25
	8	2.00	2.14
	10	1.96	2.13

TABLE IIIb

Experiment No.		R.P.M.	Ratio between the two torques
II. 106	Water	Average	4.40
	Ovalbumin	10	4.36
		20	4.38
		30	4.36
II. 108	Water	Average	4.05
	Myosin	4	4.00
		8	4.30
		10	4.25

would decline in a series of jerks, yet this again does not happen. We may have to have recourse to explanations involving high viscosity liquid crystalline flow or possibly plastic flow in two dimensions (*cf.* Scott Blair, 1938; anonymous, 1942). That molecules may move relatively to one another within films is known from the old experiments on saponin bubbles which assume curious shapes when the air contained within them is withdrawn (Lawrence, 1929, p. 110); and anomalous viscosity such as we have found for

protein monolayers has also been found for hydrocarbon chains in monolayers by Fourt and Harkins (1938).

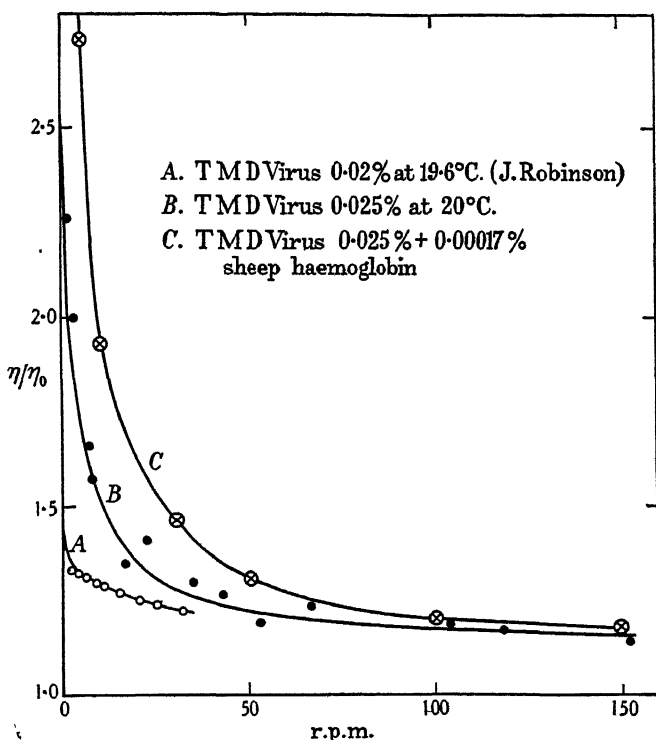


FIG. 11. Anomalous and relative film viscosity of virus nucleoprotein in presence and absence of methaemoglobin.

Relation of Optical and Viscosimetric Properties

On theoretical grounds flow-birefringence must be accompanied by anomalous viscosity—in our experience it always is. When we have found anomalous viscosity without flow-birefringence it has always been in the surface film, the thickness of which is, of course, insufficient to allow of the observation of flow-birefringence by our present methods. Nevertheless, proteins may exist which will show bulk phase anomalous viscosity without flow-birefringence; we have found mention of only one in the literature, the liver nucleoprotein of Greenstein and Jenrette (1940 *b*), whose measurements were made with capillary viscosimeters. Such cases might conceivably be due to anisometric but quite isotropic particles of the same refractive index as the medium; under such conditions flow-birefringence would not appear until they were examined in a medium of different refractive index. Or again, if the protein

particles had a tendency to linear aggregation when at rest, and if the end-to-end bonds between them were very weak, shearing might pull them apart altogether (depolymerisation) so that orientation and fall of viscosity would first be seen but flow-birefringence would never appear. It is also formally true that anomalous viscosity is a more sensitive, if less certain, method of determining the anisometry of particles than flow-birefringence, since the former method attends to the borderline of complete randomness while the latter attends to the borderline of complete orientation.

A more important point is that in our experiments with myosin, there is not an exact correspondence between the attainment of the minimal viscosity plateau and the maximal birefringence plateau. In a 0.1 per cent rabbit myosin sol at neutral pH in the viscosimeter, for example, the relative viscosity will fall to its minimal plateau level by about 6 R.P.M., but the flow-birefringence will not be accurately readable under about 30 R.P.M.

Two possible types of explanation suggest themselves for this fact. It may be that the particles of the myosin sol at rest resemble a bunch of pencils held together by an elastic band. Shearing stress first orients them, until an apparent minimal viscosity plateau is reached; but further shearing stress then pulls out the particles by making the molecules slide over one another until maximal particle length has been attained; this corresponds to the birefringence maximum. Factors which will make the molecules slide on one another would thus acquire considerable importance, having a significance similar to that "of plasticisers," *e.g.*, camphor added to celluloid, or dibutyl phthalate to synthetic resins. It is possible that the effect of adenosine triphosphate, described in the third paper of this series (Dainty *et al.*, 1944), might be of this kind. Alternatively, the myosin particles may be aperiodic coils, which shearing stress will first orient, and then proceed to stretch out.

The second type of explanation would suggest that the main part of the anomalous viscosity is due to intermicellar forces which are broken by the shear stress. In this case the minimal viscosity plateau would be only apparently parallel with the abscissa and would really be falling imperceptibly until the shear force corresponding to maximal birefringence is reached. We have not observed any signs of such behaviour.

After we had formulated the former "parallel aggregation and disaggregation" hypothesis for myosin, we found that it had already been suggested for the very curious case of sodium caseinate by Nitschmann (1938) and Nitschmann and Guggisberg (1941). Sodium caseinate is exceptional in the opposite sense to Greenstein and Jenrette's liver nucleoprotein, for it shows flow-birefringence without anomalous viscosity. Its lack of anomalous viscosity had been noted long ago by Rothlin (1919). Such behaviour could be explained if the protein consisted of rather short micelles, not very orientable at the low shear rates of anomalous flow measurements, but capable of great exten-

sion and hence becoming highly asymmetric at the higher shear rates of flow-birefringence measurement.

A rather strong argument in favour of the conception of the micelle reversibly extensible either by parallel sliding units or by coiling and uncoiling springs, is that Wöhlisch and Belonoschkin (1936) using the Gans effect (depolarisation of the Tyndall beam) could not find any great difference between the globular corpuscular and the fibrillar corpuscular proteins. It is true that the average depolarisation constant (θ_0) for myosin and ovoglobulin was greater than for ovalbumin and serum globulin (0.039 and 0.045 as against 0.025 and 0.031 respectively), but the scatter of readings showed much overlap (ranging from 0.030 to 0.052 for the two former proteins and 0.020 to 0.056 for the two latter), and none approached the value for the inorganic V_2O_5 sol of 0.131. The authors themselves pointed out that their measurements were done on myosin sols at rest, and suggested that under shear force an *Entknäuelung* or unravelling takes place.

So also the differences between the two types of protein as shown in the freezing speed method (Freundlich and Oppenheimer, 1925) were in the same direction as indicated by flow-birefringence studies, but of a very small order. Elongated particles accelerate, and spherical ones retard, the speed of propagation of ice formation in a supercooled liquid, as against pure water. But Püllen (1933) found a retardation of only 15 per cent for serum albumin, ovalbumin, and myogen, and an acceleration of only 2 per cent for myosin and ovoglobulin, though deviations of up to 50 per cent were expected. Here again the measurements were made on sols at rest. But that myosin micelles *are* to some extent elongated when at rest is not in doubt, since anomalous viscosity measurements start from rest.

The principal theoretical and quantitative treatment of the unravelling factor is that of Sakurada (1938) but it was applied only to celluloses and isoprene polymers. The proteins still await precise treatment from this point of view.

Interference by Other Proteins

It is interesting to consider whether the simultaneous presence of other, relatively spherical, protein particles, exerts an inhibitory influence on the appearance of flow-birefringence and anomalous viscosity. On thermodynamical principles, such a spherical particle should knock an anisometric particle into stream line orientation for every one which it knocks out. But the possibility of intermicellar attachments (Pedersen, 1940) warns us that the relatively spherical particle might form with the anisometric particle a combination which, even though loose and transient, would give the whole an unorientable configuration.

Empirically, there is a marked effect. Workers on virus nucleoproteins (*e.g.*,

Pirie, 1939) have informed us that only a small admixture of non-birefringent protein greatly reduces their flow-birefringence. The subject requires a specialised investigation, but we have carried out a few experiments which are worth mention. For example, a 0.63 per cent TMD virus solution (of the preparation used at that time by us) gave a flow-birefringence in the microscope cell of 145° , but on being tested at the same concentration in the simultaneous presence of 0.17 per cent crystalline methaemoglobin (which, as will be seen later, shows evidence of approximately spherical particles), the flow-birefringence fell to 95° . This was, of course, a bulk effect.

Similar effects were found for film viscosity at low shear rates. Fig. 11 shows an experiment in which the relative viscosities of TMD virus are plotted, in the presence and absence of crystalline sheep methaemoglobin. Curve *A* describes the data of Robinson (1939) for the bulk viscosity of 0.02 per cent virus at 19.6°C ., with a shear rate of 30, as against our 7.7. Curve *B* describes our data for 0.025 per cent virus at 20°C . in the film, and Curve *C* the same in the presence of 0.00017 per cent methaemoglobin. It will be seen that whereas the sample of TMD virus alone reaches its minimal level at approximately 50 R.P.M., the sample of virus mixed with methaemoglobin does not reach it till perhaps 90 R.P.M. It does therefore seem likely that the presence of even a small amount of protein of which the particles are roughly spherical will interfere with the orientation of elongated protein particles in the stream lines.

The behaviour of the strongly flow-birefringent polymerised sodium thymonucleate in the presence of other substances (Greenstein and Jenrette, 1941, 1942; Greenstein, 1942) forms an interesting parallel with the above. Most proteins and amino acids added to thymonucleate cause a small fall in its flow-birefringence and its anomalous flow, but with some organ extracts (liver, tumour, milk, serum, and even plant tissue) the fall is prolonged and severe. Since this effect can be inhibited by prior heating of the organ extracts, Greenstein and his collaborators, assuming a disaggregation, speak of a "thymonucleo-depolymerase." Their results seem, however, also to be compatible with a change in shape of the elongated micelles so that they become less orientable, either by a reduction of axial ratio by contraction or by the formation of clumps. The effects do not appear to be reversible.

DISCUSSION

It will be more profitable to postpone the general discussion of the effects of shear on protein solutions until after the specific results have been described—the reader is therefore referred to the discussion in the following paper.

The opportunity may, however, be taken here of saying a few further words about the problem from which the whole of the present work originated, namely

the change in shape of the neural cells during the formation of the neural plate in the amphibian embryo. It is at present impossible to identify the factor which is most important in these cell shape changes; it may involve (a) the fibrillar micelles in the cytoplasm, (b) the structure of the ectoplasmic layer, (c) the structure of the cell membranes.

The fibrillar micellar "cyto-skeleton" is proving difficult to demonstrate precisely, either by granule movement observations (Howard, 1932) or by orientation of cell inclusions (Pollister, 1941; Waddington, 1942) or by polarisation microscopy (Hobson, 1941) though Cowdry (1914) describes the filamentous mitochondria in the neural cells as oriented parallel to their long axes, and Waddington and Picken (1941) have reported birefringence in the piriform cells of the amphibian blastopore. At the same time we are bound to admit that something of the kind must be present, since cytoplasm shows unmistakable viscous anomaly (Pfeiffer, 1937) and thixotropy (Fauré-Fremiet, 1930, 1934). It may be that the ectoplasmic layer is responsible for the cell shape changes, for its important rôle in cell division has long been recognised. Here experiments with high pressures (see Cattell, 1936) might help us, for Marsland (1939) found that high pressures (*e.g.* 600 atmospheres) liquefy the ectoplasmic layer in the dumb-bell-shaped cleaving egg, causing recession to the spherical form; significantly, high pressures also liquefy gelatin gels (Posnjak, 1912) which are known to be thixotropic (Freundlich and Abramson, 1927). On the other hand, Brown, Hamburger, and Schmitt (1941) who have recently shown by density measurements that the early local hydration theory of Glaser for neurulation is unlikely to be true, incline to the view that the cell surfaces are of prime importance, the "attractive" forces between molecules in the adjoining cell surfaces of prospective neural tissue cells increasing so that the area of contact is actively increased. These views, which coordinate with the fundamental work of Holtfreter (1939) on the surface "affinities" of embryonic tissues, are elaborated in an interesting review by Schmitt (1941) with special reference to lipoprotein complexes. Waddington (1942) has since pushed the matter a little further by determining the surface tension necessary to spread amphibian embryo cells into a surface film; this varies with different parts of the embryo at different developmental stages.

As will be seen below, our contribution to the question lies in the fact that in the amphibian embryo there is a protein or group of proteins in the total euglobulin class which spreads instantaneously into a surface film having the property of anomalous flow. Its molecules must therefore readily pass into the fibrillar state. The evidence for this will be found in the following paper (Lawrence, Miall, Needham, and Shen, 1944). The union of all these facts into a coherent picture of morphological change at neurulation is, however, a matter for the future.

SUMMARY

1. A coaxial viscosimeter which permits the simultaneous determination of relative and anomalous viscosity and of flow-birefringence is described. Flow-anomaly and flow-birefringence are regarded as characteristic of elongated micelles and molecules.

2. Such methods have been applied to dilute solutions of proteins. The conditions under which the coaxial (Couette) viscosimeter measures the viscosity of the bulk phase and the surface film phase respectively have been investigated and are described.

3. The general behaviour of protein solutions subjected to shear is summarised.

The perennial inspiration of Sir F. G. Hopkins, O.M., must first be recorded. The authors also wish to take this opportunity of expressing their indebtedness to Professor C. E. Tilley and Dr. C. Phillips for the loan of the polarisation apparatus used, and to Dr. J. F. Danielli and Professor J. D. Bernal, F.R.S., for their friendly interest and the benefit of their conversation. Calculations concerning bulk and film viscosity were kindly made by Dr. D. J. Crisp and Dr. Danielli.

REFERENCES

The references in this paper will be found together with those of the next at the conclusion of the second paper of this series.

STUDIES ON THE ANOMALOUS VISCOSITY AND FLOW-BIREFRINGENCE OF PROTEIN SOLUTIONS

II. ON DILUTE SOLUTIONS OF PROTEINS FROM EMBRYONIC AND OTHER TISSUES

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INTRODUCTION

In the foregoing paper a description has been given of apparatus whereby the flow-birefringence and the relative viscosity of a protein solution may be measured and its anomalous viscosity assessed, careful distinction being made between effects due to the bulk phase and those due to the surface film at the air-water interface. We have now to turn to the more specific detailed results of the investigation of a number of proteins.¹

Behaviour of Proteins

Tobacco Mosaic Disease Virus Nucleoprotein.—This plant virus protein,² (for full descriptions of which see Bawden, 1939; Bernal and Fankuchen, 1941) has already been subjected to measurements of viscosity and birefringence, in the work of Robinson (1939). The specimen used by us gave the flow-birefringence curves seen in Fig. 1; allowing for differences in conditions (in our experiments 1.5 cm. column as against 21 cm.; 0.5 per cent as against 0.02 per cent concentration of virus; shear rate 13.1 as against 18.8; Robinson's sample was more flow-birefringent than ours. In the viscosimeter it was interesting to find that under both low level (film) and flood level (bulk) conditions, the virus gives a strongly anomalous type of flow, see Figs. 2 and 3.) This probably means that the virus aggregates retain their anisometric shape when they enter into the formation of the surface film.

The only difference is that the viscosity curve of 0.025 per cent virus for the bulk descends to its orientation plateau by about 20 R.P.M. while that for the film does not do so until a speed of between 70 and 80 R.P.M. is attained. Orientation within the film must therefore be a good deal more difficult than orienta-

¹ The present work was begun in 1938-39 by Joseph Needham and Shih-Chang Shen (Fellow of The Rockefeller Foundation) with A. S. C. Lawrence as rheological adviser. Margaret Miall joined the group with a Rockefeller Foundation grant in 1941. A preliminary report has already appeared (Lawrence, Needham, and Shen, 1940).

² Hereinafter called TMD virus.

tion in the bulk, as would be expected if considerable mutual interference occurred there. So far as the bulk phase is concerned, our data for TMD virus are in agreement with those of Robinson (1939) on the coaxial, and Frampton (1939) on the capillary, viscosimeter.

In the course of further experiments, it was noticed that when samples of TMD virus had deteriorated by ageing (Δ in the microscope cell having dropped

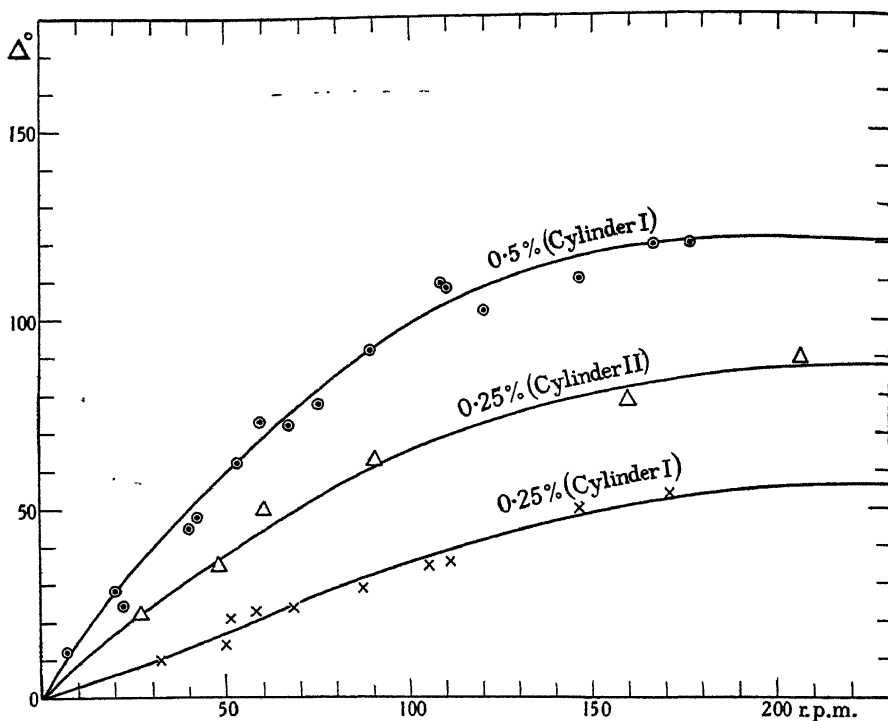


FIG. 1. Flow-birefringence (in the microscope cell) of virus nucleoprotein.

from 80° to 20° for 0.5 per cent solution at 70 R.P.M.) or if samples of fresh virus were "denatured" by the addition of a few particles of copper carbonate causing similar falls of flow-birefringence intensity, anomalous flow was no longer obtained, either in the surface film or in the bulk. Flow became normal or Newtonian and the lines describing sets of deflection readings rose unmistakably from the origin. But at the same time it also became noticeable that under film conditions (low level cylinder position) the phenomenon of anomalous return was now obtained, indicating that a built-up multilayer was being made. In explanation, it may be suggested that these forms of denaturation involve the association of the elongated virus particles into close tangles; these are unable to suffer orientation in the bulk but enter into complex relationships

within the film. It is possible, however, that denaturation involves the breakdown of the long virus particle into shorter, more nearly isometric, lengths, and then film formation reaggregates them, perhaps in some different way.

Crystalline Ovalbumin.—Crystalline ovalbumin was prepared according to the method of Larosa (1927).

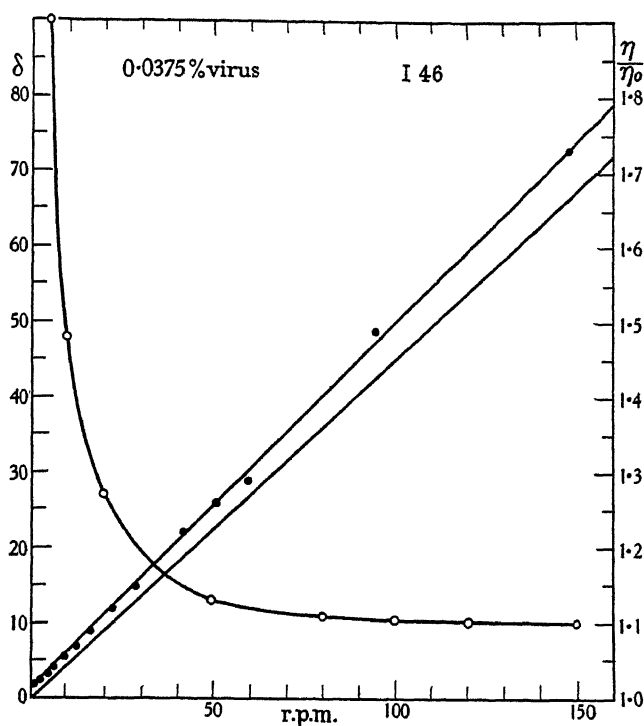


FIG. 2. Anomalous and relative film viscosity of virus nucleoprotein.

When placed in the viscosimeter, whether at low level (film) or at flood level (bulk), normal flow was invariably observed. The absence of any appreciable elongation in the native ovalbumin particles was of course expected in the light of previous knowledge of this protein (*cf.* Böhm and Signer, 1931). But this protein strongly showed the phenomenon of anomalous return, the building up of a solid multilayer (see Fig. 4). In this particular case the concentration of the protein was 0.0283 per cent.

The main interest in these built-up films lies in the fact that after they have been fully developed, it is possible to show by the present methods that elongated particles have been formed in them. Two experiments may be described which illustrate this. In the first (Experiment I 36/28d) a sample of oval-

bumin (0.0565 per cent) placed in the viscosimeter at 20°C. showed first perfectly normal flow, but as the measurements proceeded, showed an anomalous return. The solution was then taken out, stirred, filtered, diluted by half, and returned to the viscosimeter, again at low (film) level. The result was a clearly anomalous flow curve (Fig. 5), with an approximation to complete

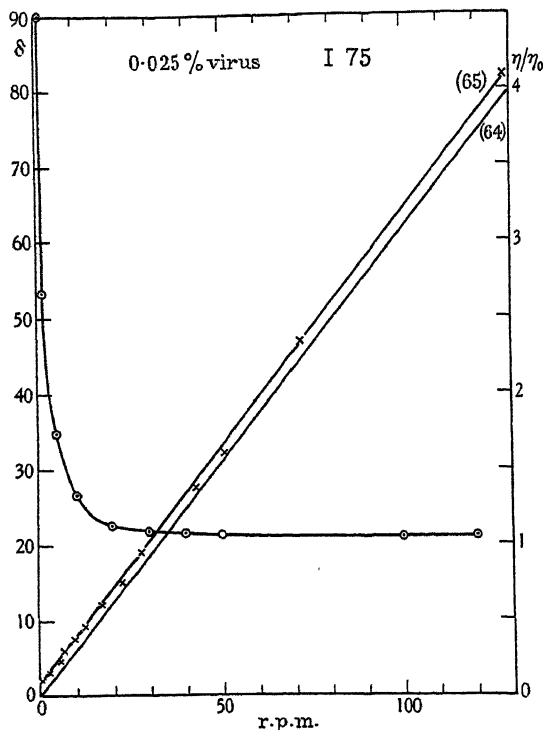


FIG. 3. Anomalous and relative bulk viscosity of virus nucleoprotein.

orientation at 30 R.P.M. Fresh ovalbumin solution measured immediately afterwards as control gave perfectly normal flow.

The second experiment shows that these artificially formed anisometric particles can be detected within the bulk also (Experiment II 103). A 1 per cent solution of crystalline ovalbumin was placed in a Langmuir trough and after the surface film had been repeatedly compressed and released, the film was drawn off, shaken up in water, filtered, and placed in the viscosimeter at flood level. As is shown in Fig. 6, anomalous flow was obtained, the only difference from the previous experiment being that the film had been longitudinally compressed rather than rotationally spun, and that (as has been seen to be the case with virus) the anisometric particles are more easily oriented in

the bulk phase than they are in the film (plateau reached at 10 instead of 30 to 40 R.P.M.) doubtless because of the lack of mutual interference there.

Some experiments were made on the behaviour of ovalbumin denatured; *e.g.*, with guanidine. If a solution of this protein is mixed with this base (in

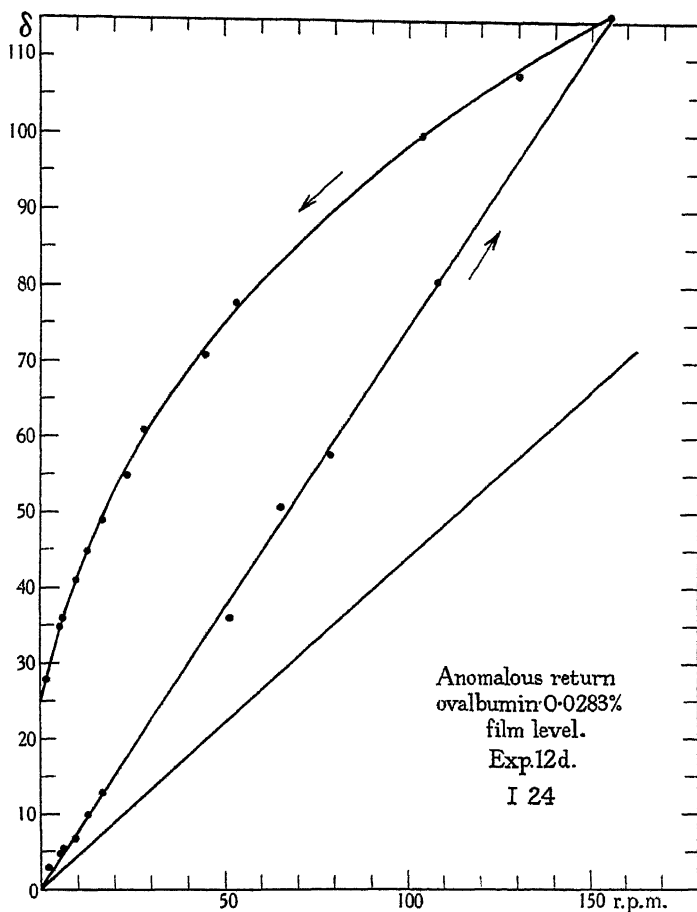


FIG. 4. Anomalous return of ovalbumin at film viscosity level.

the form of its neutralised hydrochloride) the phenomenon of anomalous return ("stretching") is much decreased or abolished. Fig. 7 shows the failure of guanidine-treated ovalbumin to build up the usual film (1 volume 0.05 per cent ovalbumin with 1 volume 3.65 M guanidine HCl). This may perhaps be interpreted by supposing that tangles of unrolled denatured molecules may be formed in the presence of guanidine, which cannot then be easily built into the usual type of film.

Spontaneous decreases of relative viscosity were often observed with this protein (*cf.* Hardy, 1905; Pauli and Valkó, 1933, p. 266).

Myosin.—Myosin was prepared from muscle tissue of rabbit and frog according to the standard methods (Edsall, 1930; Bailey, 1942). If the preparation is a good one, showing flow-birefringence, the viscosimeter behaviour is invariably the same, whether at low (film) level or flood (bulk)

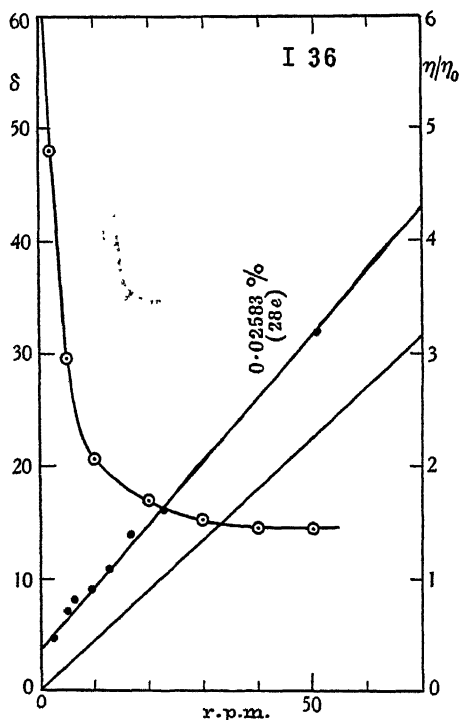


FIG. 5. Anomalous flow of ovalbumin (film) after rotation at film level.

level; it shows marked anomaly of flow. Illustrations are not given here, for the results closely resemble those shown for TMD virus in Figs. 2 and 3. Details differ, thus in one experiment with myosin at low level (Experiment I 17/6b) the protein concentration was 0.0009 per cent in *M* KCl and orientation was attained at some 70 R.P.M.; while in an experiment with myosin at flood level (Experiment II 99/82b) although the protein concentration was as high as 0.26 per cent in *M* KCl, orientation was attained already at 30 R.P.M. This brings out well the lack of mutual interference in molecular orientation in bulk as compared with film phase. An illustration of the anomalous flow of myosin has, moreover, already been given in the previous paper (Fig. 10)

in connection with the distinction between bulk and film viscosity. Since further illustrations and much further detail about myosin will be given in the succeeding paper of this series, little more will be said here. It may, however, be added that in poor or ageing preparations of myosin which show no flow-birefringence, flow anomaly may also be absent, and in this case "stretching" and anomalous return may be found; we established that in this case, just as in the case of ovalbumin, replacing the solution in the viscosimeter after the surface film had been fully formed, resulted in evidence of elongated particles by anomalous flow, both under bulk and film conditions. It would appear,

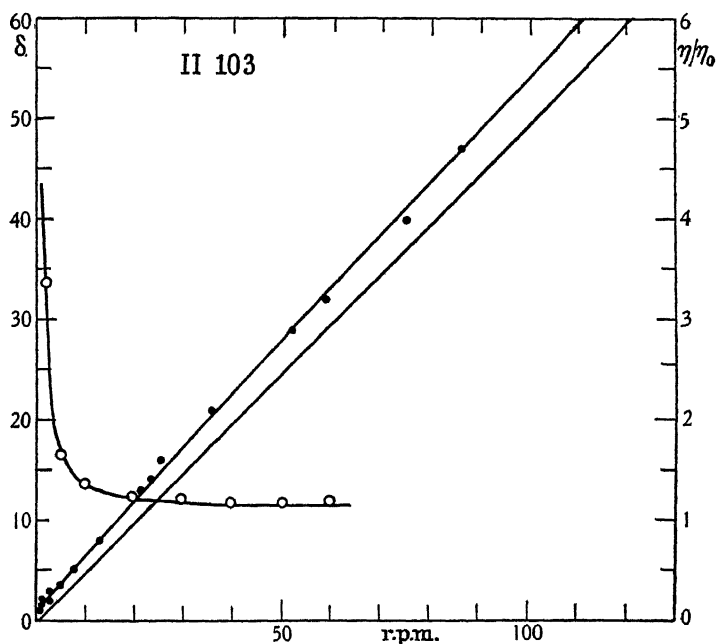


FIG. 6. Anomalous flow of ovalbumin (bulk) after compression in trough.

therefore, that the roughly spherical particles of such preparations may be made to form elongated particles again, either by being unrolled in the film or by being aggregated together in some linear manner. This re-elongation is perhaps analogous to the re-elongations accomplished by exposure of the non-flow-birefringent protein to 37°C., to be described in the succeeding paper. Myosin particles freshly isolated under the best conditions, however, never show any of these phenomena, but simply give repeatable anomalous flow whether in film or bulk.

On treatment with copper carbonate, the flow of virus nucleoprotein passes from anomalous to normal (see p. 234 above); on denaturation with guanidine

the flow of embryo euglobulin does the same (see p. 245 below). Similarly, myosin treated with N HCl forms a gel, and when brought back again to pH 8, its flow is found to be entirely normal (Experiment II 129). This is taken to mean that clumps, roughly symmetrical, have been formed from the anisometric fibrils.

Crystalline Insulin.—The insulin used was a commercial sample, tested in the viscosimeter at 0.025 per cent concentration and 20°C . Its flow in the

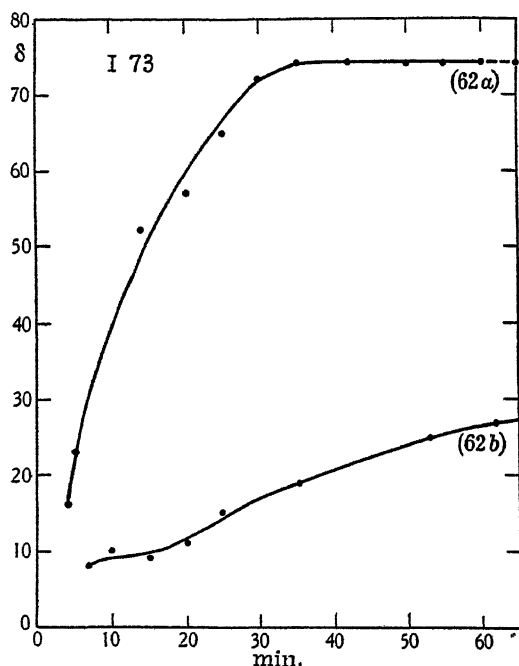


FIG. 7. Failure of ovalbumin denatured in bulk with guanidine to show "stretching" effect; building of multilayer.

surface film was perfectly normal, sets of deflection points giving lines arising unmistakably from the origin. This is in agreement with expectation.

Crystalline Methaemoglobin.—Haemoglobin used was prepared from sheep blood according to the method of Adair and Adair (1934), and converted to methaemoglobin by dialysis against tap water and distilled water at room temperature. Tested at various concentrations, of which the most suitable was 0.0002 per cent at 20°C ., its flow in the surface film was perfectly normal, as with insulin.

Ovoglobulin.—The "ovoglobulin" fraction of the hen's egg white was found by Böhm and Signer (1931) to consist of highly anisometric particles and

considered by them to account for certain properties of fresh egg white such as its very anomalous viscosity first noticed by Rothlin (1919). There has, however, been doubt as to the existence of this protein, and though it is still certainly very difficult to purify from mucins, etc. (*cf.* Needham, 1942, p. 8, 9) it does in all probability exist as a separate entity. We prepared samples for the present purpose by following closely the procedure of Böhm and Signer, which essentially consists in homogenising fresh egg white and collecting the precipitate which forms on half-saturating it with ammonium sulphate. The salt is then dialysed away and the protein purified by reprecipitation.

Tested at low level (film conditions), this protein gave markedly anomalous flow (Experiments I 64/57 and II 83/69b; 0.0212 per cent protein at 20°C.). It was noticeable that exceptionally high speeds—100 R.P.M. and above—were needed before the minimum plateau of relative viscosity corresponding to full orientation was attained.

Tested at flood level (bulk conditions), however, ovoglobulin gives no trace of anomalous flow (*e.g.* Experiment II 83/69a; 0.212 per cent protein at 20°C.). This fact throws doubt on the existence of elongated particles in solutions of the undenatured protein but suggests that as soon as denaturation at the air-water surface takes place, long molecular fibrils are formed. It would agree with this view that we were unable to detect any flow-birefringence in our ovoglobulin preparations.

Serum Globulin.—The globulin fraction of ox serum was prepared following the details in Adair and Robinson (1930), Elford and Ferry (1934), and the review of Cohn (1941). Repeated precipitation by half saturation with ammonium sulphate followed by dialysis through cellophane against weak phosphate buffer solution gave the euglobulin fraction, readily separable from the pseudoglobulins which remain unprecipitated in the supernatant liquid within the dialysis bag.

The results of viscosimeter tests showed a behaviour very similar to that of ovoglobulin (*e.g.* Experiment VI 257). Perfect and repeatable normal flow diagrams were obtained if the test was made at flood level (bulk phase viscosity) while equally definite anomalous flow diagrams were obtained if it was made at low level (film phase viscosity). This must be taken as strong evidence that the globulin particles are not elongated when in bulk solution, but immediately become so when denatured in the surface film. The diagrams closely resemble those already given in this paper (see Fig. 4) except that the relative viscosities were in all cases rather high, higher concentrations than usual being used, with 0.75 M KCl as medium.

The pseudoglobulin fraction also behaved in the same way (*e.g.* Experiment VI 260).

Plasmosin.—The study of tissue globulins, by which we mean tissue proteins soluble in strong salt solutions but not in water, has lagged much behind the

study of the globulins of the body fluids. Such proteins have, however, been examined (Bensley, 1938; Bensley and Hoerr, 1934) and their possible structural importance in cell architecture emphasised (Banga and Szent-Györgyi, 1940). We have made preparations of "plasmosin" from rabbit liver and kidney, but so far have not been able to devote to them the elaborate investigation which they require. We could, however, confirm the descriptions of them given by Bensley. In the case of the liver, we proceeded as follows:—

The perfused blood-free tissue is ground up with sand, squeezed through muslin, washed at 0°C. with 0.85 per cent NaCl, and centrifuged; this is repeated ten times or until no more protein is detectable in the washings. On mixing the residue with about five times its volume of 10 per cent NaCl the majority of the protein slowly dissolved, giving a very slimy opaque liquid. After centrifuging at 3500 R.P.M. for 25 minutes it was thrown into ten times its volume of distilled water. Fibrous filmy strands immediately formed which could be gathered about with a glass rod; these were not intrinsically birefringent under the polarising microscope. After some time, the strands contracted and cohered in the form of a clot, just as Bensley describes.

On dissolving this material in 10 per cent NaCl and centrifuging, a solution was obtained which showed no flow-birefringence and in the viscosimeter at flood level (bulk phase viscosity) gave a quite normal flow diagram. Tests were unfortunately not made at low level to detect the properties of the film phase viscosity. When these are made it will probably give anomalous flow, like ovoglobulin and serum globulin. It seems, at any rate, that by one form of denaturation, elongated particles are obtained, for Banga and Szent-Györgyi (1940, 1941), who extracted the residue from the weak salt extraction with Edsall's fluid (0.6 M KCl, 0.01 M Na₂CO₃, and 0.04 M NaHCO₃) in presence of 30 per cent urea, obtained a viscous, thixotropic solution which showed intense flow-birefringence. We were not ourselves able to repeat this observation, with plasmosin either from liver or kidney, but the solutions were difficult to deal with on account of their high light-absorption, and the subject calls for further investigation.

Mucoprotein.—We have examined mucoprotein samples from the human umbilical cord (Wharton's jelly) and from the jelly surrounding anuran amphibian eggs.

The study of mucoproteinases (hyaluronidases) has in recent years given rise to a large literature, most of the phases of which may be followed in the reviews of McClean (1933, 1941). They are of importance in connection with bacteriology, with the physiology of spermatozoa, and with the spread of substances along the skin in mammals. It is now generally accepted since the work of Chain and Duthie (1940) that the "spreading factor," as it was formerly called, is a mucolytic enzyme which attacks the polysaccharide prosthetic group of the mucoprotein molecule (an equimolecular compound of glucuronic acid and N-acetyl-glucosamine). Favilli, McClean, and Hale (1940) point

out, however, that although enzymes of this type cause an immediate and severe fall in the viscosity of their substrates, only a trace of reducing substance can be detected after 4 hours, and the maximum of free glucosamine is not reached till after 24 hours. It was therefore thought worth while to investigate the original mucoprotein preparations in the coaxial viscosimeter, with a view to ascertaining whether perhaps the fall in relative viscosity might be associated with a change in particle shape from anisometric to spherical.

We had two samples of umbilical cord mucoprotein. Sample A was a solution, an extract of 100 gm. dried cord with 2 litres water. Sample B was a dry powder, the former extract having been precipitated with 3 volumes of alcohol in the presence of sodium acetate, washed with acetone, and dried over phosphorus pentoxide, for 18 hours.

We examined the mucoprotein preparations in the viscosimeter at flood level. Neither of them showed the least trace of flow anomaly. But their relative viscosity was very high, thus that of sample A at 0.142 per cent concentration (Experiment II 123) was 15.8 times water and that of sample B at 0.041 per cent (Experiment II 125) was 4.4 times, (all at 20°C.). No trace of flow-birefringence was observable. It is highly probable, therefore, taking all these facts into consideration, that anisometric particles are absent from these preparations and the sharp fall of viscosity which the enzyme brings about must rather be due to decreases in intermicellar forces or some similar factor. The mucoprotein solutions were, however, more stable than any other preparation which we encountered, and preserved their viscosimetric properties unchanged over a long period.

A similar result was obtained with the mucoprotein of amphibian egg jelly. We took advantage of the fact long known to experimental morphologists that anuran jelly is soluble in strong potassium cyanide solution. To 80 cc. of 10 per cent KCN were gradually added 370 cc. of cleaned egg jelly; the final volume was 447 cc. and the KCN concentration 1.8 per cent. The filtered liquid was clear and syrupy. The mucoprotein was precipitable with excess of alcohol but not with acetic acid. In spite of its appearance, the solution was not markedly more viscous than a myosin sol (η/η_0 1.58) and its flow was perfectly normal (Experiment II 115). (It should be noted, however, that the treatment of ovomucoid with KCN may bring about changes in the structure of the protein.)

It is unfortunate that no tests were made of these proteins at low level for film viscosity, for it is likely on histological grounds that mucoproteins may have something to do with fibril formation, and this may involve a kind of denaturation.

Behaviour of Proteins from the Amphibian Embryo

As mentioned in the introductory paragraph of this paper, our interest in the study of protein particle shape was originally aroused by the profound changes

in cell shape which occur in the vertebrate embryo when the cuboidal ectodermal cells and nuclei are elongating to form the constituent cells of the neural plate and tube. In view of the central position which the amphibian embryo has played for many years in the study of causal morphology, it is remarkable that no attention whatever has been paid to its proteins. For the experiments here described we used the embryos of the common frog, *Rana temporaria*, and toad, *Bufo vulgaris*. At first we were careful to use only embryos at the neurula stage, but finding later that the distribution of the proteins does not seem to suffer great changes during development, we also used unfertilised and fertilised eggs, and the earliest hatching stages. Removal from the jelly was accomplished either by dissolving it in strong KCN solution or, more commonly, by snipping the embryos out with scissors, or by allowing hatching to occur and then collecting the free swimming larvae from the jellies, which, with suitable skill, is an easy matter.

Fractionation of the embryo tissue proceeded as follows: The wet material was frozen solid in a vessel surrounded by ether and solid CO₂; *i.e.*, at a temperature of about -77°C. and stored over phosphorus pentoxide, calcium chloride, or anhydrous aluminium chloride at a vacuum of 0.01 mm. Hg at 0°C. till needed. Since it was sometimes desired to free it from as much fatty material as possible, it was found that by repeated treatments at -77°C. with pure absolute alcohol and anhydrous sodium-dried ether successively, a very large part of the lipoidal constituents and greenish-yellow pigments may be removed without denaturing the proteins. When required for use, the black powder was ground up with fine quartz sand and M KCl, the several extracts mixed together, and centrifuged at 11,000 R.P.M. for about 20 minutes, ice water being run through the Ecco centrifuge-cooling system. The tube will now show the black melanin granules packed together at the bottom while above the yellowish opalescent aqueous solution there is a cap of fat which can usually be removed entire with a ground glass rod or wood splinter fragment. On pouring this solution into ten times its volume of strictly neutral distilled water, the total euglobulin comes down as a voluminous creamy-white precipitate, while the pseudoglobulin remains in solution. Various methods of fractionating the total euglobulin, which must consist largely of vitellin, have been tried, and will be described below. The black residue from the KCl extract and the separated melanin will then yield a small amount of protein to direct aqueous extraction, and a further small amount to extraction with alkaline buffer.

The distribution of nitrogen among these fractions (Table I) clearly shows that much the greatest part of the embryo protein (72 per cent) is in the total euglobulin fraction. Our special interest in the neural tube region led us to separate, in most of the earlier experiments, the neural part from the ventral part of the embryo, but, as Table I shows, the differences between them, as far as this rather crude analysis goes, were insignificant, and later we used the whole embryo throughout.

Total Euglobulin.—Early in the work it was found that when the total euglobulin fraction is examined in the viscosimeter at low level (for film viscosity) it invariably gives diagrams showing anomalous flow, and it is not “stretchable;” *i.e.*, anomalous return is never observed. Since these observations are important, a typical diagram (Experiment I 51/42b for 0.00084 per cent protein at 20°C.) is given in Fig. 8. The slow fall of the relative viscosity, not attaining its minimum plateau till 100 R.P.M. or so, characteristic of film flow anomaly, will be noted. No differences were ever observed between the behaviour of the euglobulin preparations from the neural and ventral portions of the embryos.

TABLE I
Nitrogen Content of Protein Fractions from the Amphibian Embryo

		Neural portion		Ventral portion	
		N/100 mg. dry weight tissue	Protein/ 100 mg. dry weight tissue	N/100 mg. dry weight tissue	Protein/ 100 mg. dry weight tissue
Total nitrogen.....		10.45		11.60	
		mg.	mg.	mg.	mg.
Total KCl extract	KS	9.69	60.50	10.98	68.50
Pseudoglobulin	A	1.07	6.68	2.77	17.30
Total euglobulin	TG	7.73	48.30	8.06	49.60
Acid-sensitive vitellin fraction	PG	3.31	20.70	8.24	51.60
Acid-resistant “myosin” fraction	SG	0.85	5.31	0.12	0.72
Fraction water-soluble after acid treatment	S	3.18	19.80	0.085	0.53
Aqueous extract of residue from KCl extract	WS	0.07	0.41	0.014	0.09
Final alkaline extract of residue	AK	0.13	0.81	—	—

When later the total euglobulin fraction was examined at flood level (for bulk viscosity) no sign of anomalous flow could ever be obtained and as it was at first thought that this might be due to the presence of lipoidal material in the preparations used, much effort was devoted to their delipidation. Eventually, however, it was found that the same sample of total euglobulin, whether delipidated or not, will regularly give normal or anomalous flow according to whether it is examined in the bulk or in the film (*e.g.* Experiments V 239–243). This fraction, therefore, behaves in a very similar way to serum globulin and ovoglobulin (and probably also the tissue globulins or plasmosins) and we must suppose that the approximately spherical particles of the bulk phase are unrolled into fibres or rods when denatured in the film.

The question of prior denaturation of the euglobulin is interesting. If it is first treated with guanidine in solution (*e.g.* Experiment I 58/51d guanidine

HCl 1.55 M, total euglobulin 0.00201 per cent) the anomalous flow is completely abolished, and the relative viscosity of the film is constant whatever the speed of rotation. This is perhaps because the particles, unrolled in the solution, aggregate into tangles which film conditions can never pull out. Such an explanation is strongly supported by the fact that if the total euglobulin is taken up into Edsall solution to which 30 per cent urea has been added, as in the experiments of Banga and Szent-Györgyi (1940), neither flow-birefringence nor any trace of anomalous flow is to be observed in the bulk phase, although

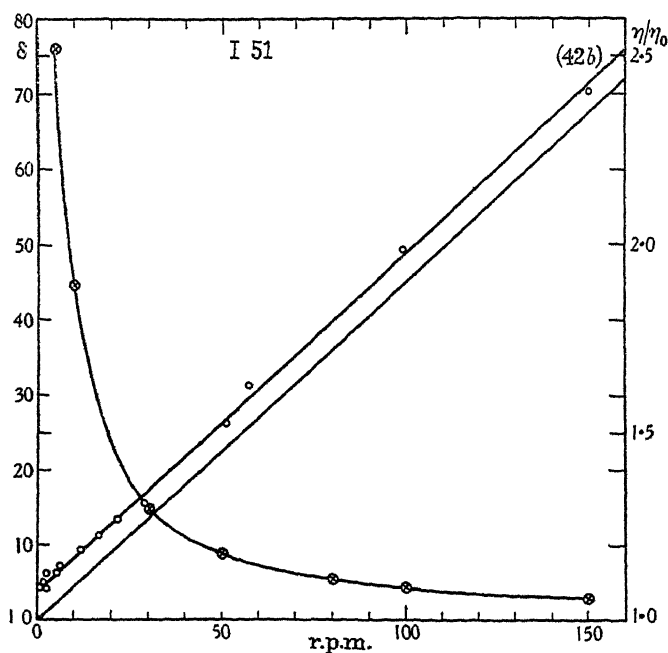


FIG. 8. Anomalous and relative film viscosity of amphibian embryo euglobulin.

the protein is certainly denatured (Experiment V 237/vi b). This is the same behaviour as that described by Banga and Szent-Györgyi (1941—abstract only available) for serum albumin, serum globulin, lactalbumin, ovalbumin, and casein.

The question next arises whether the material with which we are here dealing is wholly vitellin, or whether other globulins of greater structural importance accompany a phosphoprotein which has classically been looked upon as a mere reserve substance. It may be that this attitude to vitellin is a prejudice of which we ought to rid ourselves. We know that phosphorus is removed from it as the yolk is utilised during embryonic development (*cf.* Needham, 1931, p. 1198 *ff.*; 1942, p. 15) but we know nothing of what happens to the rest of the

molecule. There are, moreover, indications that it is capable of participation in orderly structures, for Radlkofer (1859), Schmidt (1924, p. 455, 456), and Fauré-Fremiet (1933) have described weak but definite birefringence in the innumerable yolk platelets which are so prominent a feature of embryonic cells. It may be, therefore, that vitellin is a source of fibre molecules to the embryo as well as phosphorus atoms. The yolk platelets are playing a more and more important part in modern experimental embryology, especially in regard to the mechanism of gastrulation; thus Pasteels (1940, p. 383) writes:

"La distinction faite trop souvent en protoplasme 'vivant' et enclaves 'mortes' semble spacieuse et dangereuse, car la morphogénèse peut être considérée comme le résultat d'un métabolisme complexe et changeant ou peuvent être impliqués tôt ou tard tous les matériaux de l'oeuf, sans distinction de leur état physique ou de leur visibilité microscopique."

Brachet (1940) has described a protein giving a strong -SH reaction and staining characteristically with pyronine and methyl green. It is first seen in the nucleus of the oocyte, its distribution is later coterminous with the organising region over the dorsal lip of the blastopore, and it eventually appears in the cytoplasm throughout the neural plate and tube. Brachet writes (p. 197):

"Ajoutons que des granulations colorables à la pyronine n'apparaissent à l'intérieur même des cellules qu'au moment où la neurulation débute; elles ont alors l'aspect des fibrilles orientées suivant la longueur des cellules nerveuses. On peut se demander si la présence de ces fibrilles n'a pas d'effet sur l'acquisition de la forme du système nerveux; sa structure palissadique si caractéristique ne résulte certainement pas de l'orientation de mitoses, qui est éminemment variable, mais de l'alignement des cellules. Quand on examine le problème à la lumière de nos connaissances récentes sur la structure des protéines, on est en droit de se demander si la forme des cellules nerveuses et les mouvements morphogénétiques que subit le système nerveux, ne proviennent pas de la structure et des déformations que leurs molécules protéiques sont susceptibles de subir."

Attempts to fractionate our total euglobulin fraction were therefore indispensable.

These attempts took four forms: (1) high-speed centrifuging, (2) Tiselius electrophoresis, (3) treatment with acid, (4) fractional dialysis. Up to the present time, only the fourth method has been in any way successful.

We were never able to separate the euglobulin into different fractions by high-speed centrifuging. For example, subjection of the total KCl extract to 16,000 R.P.M. for 2 to 3 hours brought down nothing, and the solution afterwards gave just as marked anomalous flow in the film as it had done before (*e.g.* Experiment I 60/53a). Owing to lack of equipment and spontaneous denaturation of the euglobulin (which readily occurs) during transit to other laboratories,

trials in the Tiselius apparatus were ineffective. This technique, moreover, is particularly difficult with proteins such as these, since they are most stable in M KCl at neutrality, while for electrophoresis ten times less salt and an alkaline pH is required.

Separation by acid treatment was suggested by the possibility that since in the neurula the somites are forming, there might be a small quantity of myosin-like globulin mixed with the vitellin. The total euglobulin fraction was simply treated with $N/100$ HCl, in which it dissolved, and after standing for varying intervals of time at different temperatures, reprecipitated by neutralisation. Only small amounts of the protein were now soluble in KCl, it being found as expected (Bate-Smith, 1937) that vitellin is denatured by this treatment. However, these soluble "myosin" fractions gave only the same anomalous flow in the film phase as the whole original fraction (*e.g.* Experiment I 52/43b; "myosin" at 0.0017 per cent at 20°C.), so not much advantage was gained. As may be seen from Table I, this "myosin" fraction is not quantitatively very important, though a good deal more of it was obtained, as expected, from the neural than from the ventral part. Another fraction was met with which, though not water-soluble before the acid treatment and therefore following the total euglobulin, remained in the neutralised solution following the acid treatment and would not go down with the denatured vitellin and the undenatured "myosin." This fraction, S, (like the "myosin" fraction itself, SG) was much more plentiful from the neural than from the ventral parts, but it resembled the pseudoglobulin (A) in giving a perfectly normal flow diagram tested under film conditions though differing from it in giving no trace of anomalous return (Experiment I 62/55b; 0.0055 per cent concentration at 20°C.). Owing to lack of material, further information about these fractions could not be obtained.

The spring of 1942 provided exceptionally abundant supplies of amphibian material, so a number of fractional dialyses at neutral pH were carried out, and these effected a better separation than any of the other methods (*cf.* Green's fractionation of horse serum proteins, 1938).

The first experiment was made as follows: About 24 gm. freeze-dried whole-embryo powder (from hatched larvae with external gills) were ground with quartz at 0°C., and delipidated. The delipidation process was the same in all experiments and consisted of three 20 minute extractions of the powder with pure absolute alcohol, followed by four 30 minute extractions with pure sodium-dried ether, all at -77°. The total euglobulin, when extracted and purified, was taken up in M KCl buffered to pH 7.1 with bicarbonate. 30 cc. of this solution were placed in each of eight sacs made from cellophane tubing, and dialysed against various concentrations of KCl so arranged as to give the final equilibria shown in Table II. The sacs were rotated mechanically for the first 6 hours and then remained at 0°C. for 10 hours to attain equilibrium. As expected, increasing amounts of precipitate were thrown out as the final salt concentration diminished; thus in A and B there was but a trace of precipitate; in C, D,

and E the precipitate was flocculent and increasingly massive; while in F, G, and H it was voluminous and creamy, ending in the usual total euglobulin precipitate.

The amounts of protein, determined by Kjeldahls, in each fraction precipitated are shown in Table II. This method has, of course, the disadvantage that each of the later fractions in the series contains all the preceding fractions. Nevertheless it was clear that a certain fractionation had been attained in this

TABLE II
Fractional Dialysis of Total Euglobulin of Amphibian Embryo

Experiment		Final molarity KCl reached by equilibration	Protein N	P/N ratio × 100	Viscosity properties of film phase
			<i>gm.</i>		
I (progressive)	A	0.74	0.005	7.65	Insufficient for test
	B	0.565	0.0115	7.07	“ “ “
	C	0.443	0.132	4.93	Denatured; no test
	D	0.32	0.228	5.16	Marked anomalous flow
	E	0.224	0.251	5.87	“ “ “
	F	0.113	0.278	5.96	Not tested
	G	0.0148	0.294	6.04	“ “
	H	0.001	—	6.20	“ “
II (separated)	A	0.454	0.1805	6.16	Marked anomalous flow
	B	0.403	0.1313	4.78	Slight “ “
	C	0.356	0.1135	5.46	Very slight anomalous flow
	D	0.303	0.076	5.40	Normal flow
	E	0.195	0.093	6.37	“ “
	F	0.090	0.018	6.44	Not tested
	G	0.009	0.0044	7.54	“ “
III (separated)	A	0.453	Trace	7.00	Insufficient for test
	B	0.399	0.052	6.88	Marked anomalous flow
	C	0.329	0.034	6.64	“ “ “
	D	0.150	0.029	7.58	Normal flow

way, for the precipitates in C, D, and E had a different appearance from the later fractions, and when the phosphorus-nitrogen ratios were obtained, showed a marked difference. As the graph in Fig. 9 shows, the P/N ratio of the small amounts of globulin very precipitable by reduced salt concentration was high, as also was that of the large amounts of globulin which only came out of solution at the low salt levels; but the P/N ratio of the flocculent protein thrown out at the intermediate levels was decidedly low. The value varied from somewhat over 0.07 to somewhat under 0.05. For purified hen's egg vitellin the accepted P/N ratio (Osborne and Campbell, 1900; Jukes and Kay, 1932) is 0.0575, but

from the only modern figure we possess for frog's egg vitellin (McClendon, 1909) its ratio would seem to be considerably higher: 0.0785. The experiment suggests, therefore, that the total euglobulin fraction consists of at least three

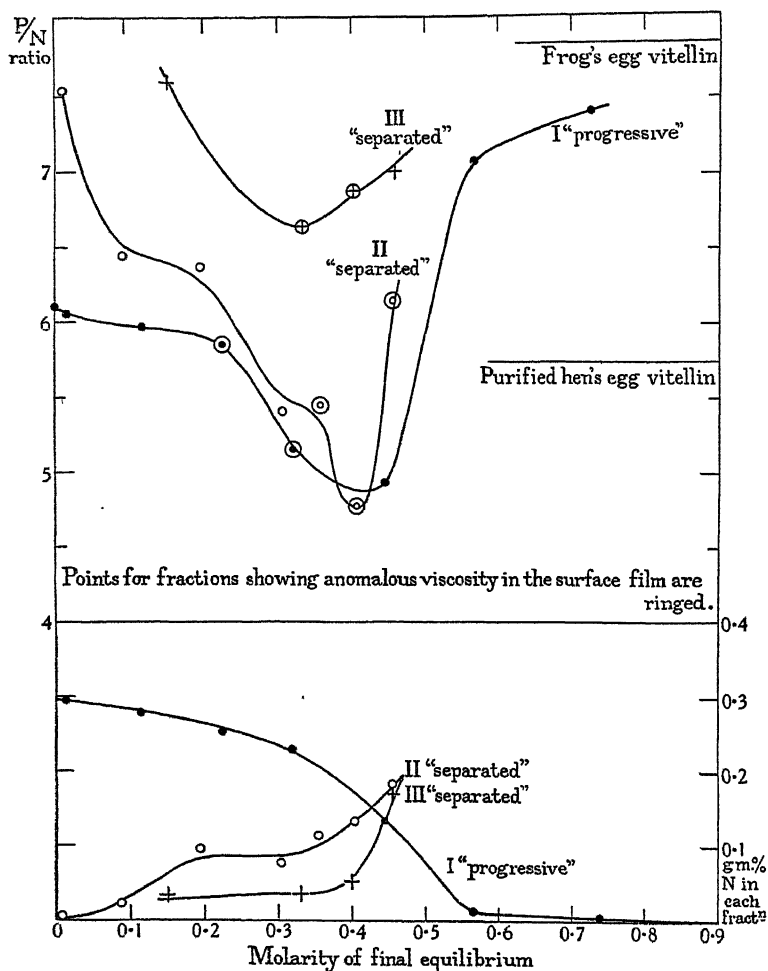


FIG. 9. Separation of fractions of amphibian embryo euglobulin by dialysis to various salt concentrations.

proteins: (1) a protein of high P/N ratio, present only in small quantity, and markedly precipitable by reduced salt concentrations, (2) a protein of low P/N ratio, present in larger amount, not so precipitable by reduced salt concentrations, and (3) a protein of high P/N ratio, present in very large amount, but not coming out of solution till the salt concentration falls below 0.25 M. We

must almost certainly identify the third of these with vitellin. In this experiment, viscosimetric observations were only made on fractions D and E; these corresponded to the second of the three proteins, and in each case gave definite anomalous flow in the film.

These interesting results encouraged us to go further, improving the method by exposing the same sample rather than a series of different samples of euglobulin to successively reduced salt concentrations, so that whatever came out of solution at each equilibrium concentration could be removed by centrifuging and separately examined. Since the critical concentrations at equilibrium seemed to be between 0.5 and 0.25 M, attention was directed especially to this range and to avoid all denaturation, every part of the operations was carried out at 0°C. As Table II and Fig. 9 show, the results of the second and third experiments were in agreement with the conclusions of the first. The starting material of Experiment II was 45 gm. embryo powder from just hatched larvae, and that of Experiment III was 6.5 gm. mixed embryo powders, mostly from neurulae. In both cases the P/N ratio first fell and then rose as the equilibrium salt concentration was reduced. But the new finding here of greatest importance was that in both cases the fractions precipitating at the lower salt concentrations (D, E, F, and G in Experiment II; D in Experiment III), and hence, on the basis of the first experiment, most likely to be vitellin itself, did *not* give the phenomenon of anomalous viscosity in the surface film. This was restricted to the central fractions (A, B, and C in Experiment II; B and C in Experiment III), associated with the lower P/N ratios.

Three fractions thus appear to be present, which we may classify and describe provisionally as follows:

- | | |
|--|---|
| Amphibian embryo euglobulin <i>a</i> . | Precipitates between 1.0 M and 0.5 M KCl concentration; present in very small amount; P/N ratio high; viscosity properties unknown. |
| Amphibian embryo euglobulin <i>b</i> . | Precipitates between 0.5 M and 0.3 M KCl concentration; present in considerable amount; P/N ratio lower; shows highly anomalous flow in the surface film. |
| Amphibian embryo euglobulin <i>c</i> . | Precipitates between 0.3 M and zero KCl concentration; present in very large amount; P/N ratio high; shows normal flow in the surface film. |

Whether these are three forms of vitellin itself, or whether euglobulin *c* is to be identified with true vitellin and euglobulin *b* with Brachet's protein; what is the nature and significance of euglobulin *a*; and whether still further fractions could be distinguished, must be left for further investigation. We have, however, the clue that Mirsky and Pollister (1942) find the plasmosins to be (cytoplasmic?) nucleoproteins, so that a close relationship may exist between

Bensley's plasmosin, Brachet's gastrular protein, and our embryo euglobulin *b* (cf. Schultz, 1941, p. 59).

It has to be remembered that delipidation in the cold, as was necessary here, is not always adequate, however prolonged or repeated. Hence small amounts of lipid may be present and affect the P/N ratio. These results must therefore be regarded as preliminary, and estimations of the actual lipid content of the fractions are necessary.

Pseudoglobulin.—The pseudoglobulin is extracted from the embryo powder by the molar salt solution, but remains behind when the total euglobulin is thrown out by dilution with ten times the volume of water. It corresponds to the livetin of Plimmer (1908) and Kay and Marshall (1928) obtained from hen's egg yolk and the corresponding pseudoglobulins which have been described from yolk of many other animals, such as the dogfish egg thuichthin of Needham (1929).

As may be seen from Table I there was rather more of it in the ventral than in the neural portion of the amphibian embryo. It was soon found to differ markedly from the pseudoglobulin of serum (already mentioned) for it never, in the first instance, shows anomalous flow in the viscosimeter at low level (film viscosity). Its normal flow lines are, however, immediately succeeded by anomalous return, and it is, indeed, one of the most eminently "stretchable" of the proteins we examined. Thus (in Experiment I 42/30ccc) at 19.5°C. with the viscosimeter running continuously at 11.9 R.P.M. the mirror deflection rose from 9 to 97 mm. in 50 minutes. It is also worth noting that this protein has more tendency to lose relative viscosity on standing at 0°C. than any other in our experience (cf. Pauli and Valkó, 1933, p. 266). Thus, (in Experiment I 47) a concentration of 0.00055 per cent protein, which to begin with had a film viscosity of $\eta/\eta_0 = 4.35$ had fallen after 8 hours to a level barely distinguishable from that of water.

In some experiments (such as Experiment I 55/50bc, in which 0.011 per cent protein was used), the solution, diluted by half, filtered, and replaced in the viscosimeter immediately after normal flow and a large anomalous return had been observed, gave straightway an anomalous flow diagram, showing presumably that the denatured fibrils of the film retained their character and were ready to form a spontaneous surface film flow anomaly. But if, instead of diluting with *M* KCl, the contents of the viscosimeter was diluted with *M* KCl containing a suitable concentration of guanidine HCl (3.15 *M* in Experiment I 59/52abc), then no anomalous flow in the film was observable upon trial in the viscosimeter. This experiment is shown in Fig. 10. It must again mean that the fibrils in the bulk phase are aggregated into tangles by the dissolved denaturing agent, and perhaps in some way still further denatured, so that they can take no part in forming a new film showing flow anomaly.

It may be concluded that the pseudoglobulin is as capable of forming surface

film fibre particles as the euglobulins, but that the process is much slower under our conditions than with euglobulin *b*, which gives the flow anomaly immediately the film is set up.

Other Fractions.—In order to try to extract mucoproteins, nucleoproteins etc. from the KCl-extracted residue, extracts were made with distilled water (Experiment I 53/44) and phosphate buffer solution at pH 9.0 (Experiment I 65/58). As Table I shows, no appreciable amounts of protein could be extracted by these means. Examined in the viscosimeter both fractions showed

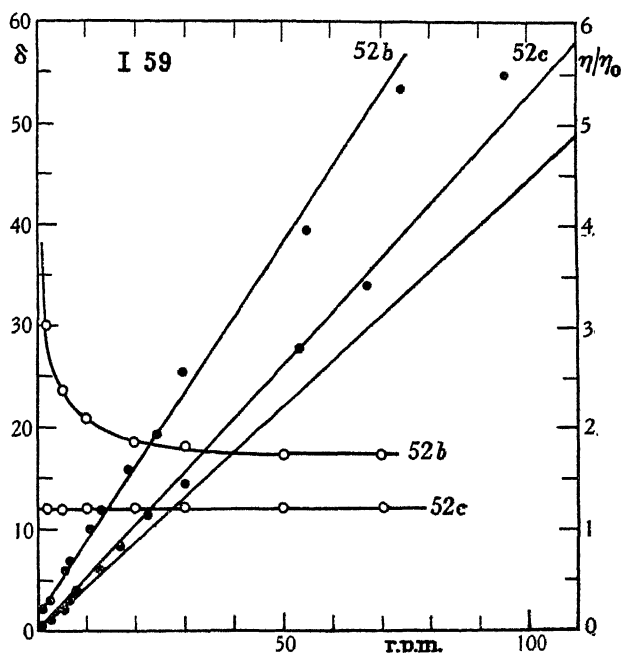


FIG. 10. Anomalous flow of amphibian embryo pseudoglobulin (film) after rotation at film level.

quite normal flow, but the amount of material available was insufficient to establish their properties further.

DISCUSSION

The data reported in this paper, the first extensive survey of the behaviour of dilute solutions of proteins in the coaxial viscosimeter, may be partly summarised in the form of a table (Table III). Group A includes the proteins clearly fibrillar or rod-like in the bulk phase as well as in the film. Probably also belonging to this group are fibrinogen (Wöhlisch and Clamann, 1932; Böhm and Signer, 1932); keratin (if in thioglycolic acid solution; Goddard and

Michaelis, 1935); lens albumoid (Böhm, 1934); the stromatin of erythrocytes, which is thought to form a lattice in the blood corpuscle (Böhm, 1935; Parpart and Dziemian, 1940; Furchgott, 1940); elastoidin (Fauré-Fremiet, 1936, 1937; Champetier and Fauré-Fremiet, 1937); collagen (Mehl, 1938; Champetier and Fauré-Fremiet, 1938); the neuronin of nerve fibre axoplasm (Bear, Schmitt, and Young, 1937); fibroin and sericin (Meyer and Jeannerat, 1939); and a plant

TABLE III
Provisional Classification of Proteins As Studied in the Coaxial Viscosimeter

A	B		C
Proteins which show flow-anomaly in bulk and in film, with flow-birefringence Fibrillar in bulk and film.	Proteins which show flow-anomaly in film but not in bulk (except after film) Fibrillar only in film (or in bulk after film)		Proteins which show flow-anomaly neither in film nor in bulk
	B ₁ . Immediately (Polyfilm formation very rapid)	B ₂ . Only after anomalous return ("stretching"). (Polyfilm formation slow)	
Tobacco mosaic virus	Mammalian serum euglobulin	Crystalline avian ovalbumin	Crystalline insulin " methaemoglobin
Myosin	Mammalian serum pseudoglobulin Avian ovoglobulin Amphibian embryo euglobulin <i>b</i> Plasmosins (?)	Amphibian embryo pseudoglobulin	Amphibian embryo euglobulin <i>c</i> (= vitellin?) Amphibian egg jelly mucoprotein Human umbilical cord mucoprotein

protein occurring in heather-honey (Pryce-Jones, 1936). This list includes the fibrillar corpuscular proteins and any fibrillar (normally non-corpuscular) proteins which can be got into solution.

Thyreoglobulin and certain haemocyanins have been stated to show flow-birefringence, but it is extremely weak (Lauffer and Stanley, 1938). Certain fractions of both the serum albumin and the serum globulin group may also be flow-birefringent (Sadron, Bonot, and Mosimann, 1939). Gelatin, as a degradation product of a fibrillar non-corpuscular protein (collagen) stands in a somewhat special position; both flow-birefringence (Umlauf, 1892) and anoma-

lous viscosity (Rothlin, 1919) have been reported for it, but in view of the difficulty of obtaining standard preparations it requires reinvestigation by modern methods.

Bulk anomalous viscosity has been observed for myosin (von Muralt and Edsall, 1930*a*, p. 340; Frampton, 1939; Edsall and Mehl, 1940), for lens albumoid (Böhm, 1934), for erythrocyte stromatin (Böhm, 1935), and for ovoglobulin (Böhm and Signer, 1931); all using flow through capillary viscosimeters at varying pressures. Other proteins tested in this way with negative results are casein (Rothlin, 1919), ovalbumin and ovomucoid (Böhm and Signer, 1931), lactoglobulin and serum pseudoglobulin (Neurath, Cooper, and Erickson, 1942*a*), haemocyanin (Polson, 1939), and myogen (Weber, 1933, p. 128).

We hope in the course of time to investigate as many as possible of these proteins with our methods.

Group B includes all the proteins tested which unfold ("stretch") as denaturation occurs within the surface film. It has long been known that a molecule of ovalbumin, having a radius of 55 Å (Adair and Adair, 1940) unrolls into a surface film of but 9.5 Å thickness (Astbury, Bell, Gorter, and van Ormondt, 1938). Some members of this group do it instantly (group B₁) giving anomalous flow diagrams in the first instance, while others (group B₂) require a considerable time to do so, and only give anomalous flow diagrams after the film has built itself up for half an hour or more. It may be surmised that the first of these groups includes many globulins and the second many albumins. The time factor in film formation has, of course, been known since the early work of Wilson and Ries (1923) and Gorter and Grendel (1928).

Finally, there is a group of proteins which has not given us at any time anomalous flow, either in bulk or film, (group C). The changes occurring in surface denaturation do not seem in these cases to lead to fibrillar particle formation, or if they do, the fibrils are not orientable. It is possible that this group would merge into group B₂ under conditions other than those used by us.

It is of much interest that the particles of proteins of group A, which are highly elongated in the bulk phase, remain so in the film. Seastone (1938) found that the virus spreads at air-water interfaces very unwillingly, but in our experiment there was no doubt that a film was present. Particles of proteins of group B, which are certainly not elongated in the bulk phase, become so in the film, and can later be detected in the bulk phase if the film is dispersed by shaking (Fig. 6). The distinction between group A and group B is probably the same as that in the now commonly used expressions "fibrillar corpuscular" and "globular corpuscular" proteins (Astbury, 1941).

Certain discrepancies still remain, however. For example, Böhm and Signer (1931) studied the proteins of hen's egg white with a coaxial apparatus similar to that used by Signer (1930) for measuring the flow-birefringence of polystyrols and Vorländer and Walter (1925) for paraffin chains, etc. Viscous anomaly was observed by timing

the speed of flow through a capillary viscosimeter at different pressures. Neither ovalbumin nor ovomucoid showed a trace of flow-birefringence or anomalous viscosity, but ovoglobulin showed both to a marked degree, accounting for the flow-birefringence and anomalous viscosity said to exist in fresh egg white. From the description of their apparatus given by Böhm and Signer it is not clear whether their cylinder was completely immersed in the protein solution or not, but it seems probable that it was. As their preparation of ovoglobulin gave very strong flow-birefringence and bulk phase anomalous viscosity it seems hardly likely that their higher shear rate as against ours (131 to 44) could have been wholly responsible for the absence of these phenomena in our preparations. We did, of course, find anomalous viscosity in the film. The subject requires further investigation but clearly ovoglobulin may belong to group A rather than to group B.

Our knowledge of the viscosity of protein surface films is not as yet very extensive. The capillary slit surface-viscosimeter and the torsion-ring surface-viscosimeter of Myers and Harkins (1937) have not so far been applied to surface films of proteins. But by measuring the damping of an oscillating platinum disc or vane suspended in the surface film by a torsion wire, it has been possible to measure the absolute viscosities of a number of protein surface films (Langmuir, 1938; Langmuir and Schaefer, 1937, 1939; Fourt, 1939). They vary over a range of about 1:10,000, typical figures being (in μ at pH 5.8 and 25°C. for $F = 6$ dynes) gliadin, casein, and zein about 0.005, up to trypsinogen 1.0, edestin 26, and serum globulin 210. But these correspond to our relative viscosities, and the *relative* viscosity of a protein, though certainly related to the degree of elongation of its particles, is not related to molecular shape in a simple way. It does not give the same conviction of elongated shape which may be derived from the observation of *anomalous* viscosity. Thus Cohn (1939, p. 223) points out that from the pioneer work of Chick (1914) and Chick and Lubrzynska (1914) to the more refined investigations of Fahey and Green (1938) it has been known that the relative viscosities are related as follows:—sodium caseinate > serum euglobulin > serum pseudoglobulin > serum albumin > ovalbumin; and this has long been interpreted as being the order in which the shapes of these molecules differ from spherical symmetry (*cf.* Pauli and Valkó, 1933, p. 240). But, as Cohn says, “a completely satisfactory theoretical equation relating the two properties remains to be developed. The problem has been repeatedly considered, and various extensions of the Einstein equation have been suggested and temporarily employed. Estimates of the relation of the two principal axes of the molecule must be adopted with caution, until they prove to be identical with those derived from diffusion measurements, dielectric constant measurements, and measurements of flow-birefringence.” Perhaps this should apply also to the calculations of Neurath (1939) who assesses the axial ratios of the majority of globular corpuscular proteins at from 1 to 10, in contrast with the well established 10 to 100 of the

TMD virus (Lauffer and Stanley, 1939; Kausche, Pfankuch, and Ruska, 1939) and myosin (Mehl, 1938). Moreover, as will be seen in the succeeding paper of this series, in different myosin samples, relative and anomalous viscosity are independent variables. A sample showing normal flow may have a higher relative viscosity than one showing anomalous flow.

This point of view is substantiated by another approach to the nature of the protein surface film, also due to Langmuir (Langmuir, 1938*a*; Schaefer, 1938). The expansion patterns made by dropping oil of known properties on to the centre of a protein surface film, itself already surrounded by an oil of known properties, reveal qualities of cohesion anisotropy in the protein monolayer (*cf.* liquid crystals). The oil spreads from the centre in the form of a sharp pointed star, opening up, as it were, and following "faults" in the protein film;³ and later, these strips of protein film, separating the oil channels, can be lifted off the surface as visible fibres. Similar fibres will also form parallel to the barrier compressing a protein film. They indicate, says Langmuir (1938*a*, p. 177) "the presence of long chain molecules." Not all protein films behave in this way, however. Some give a smooth circular boundary between the protein and the advancing oil; others, an intermediate form, give a rough circular boundary with a serrated edge. The proteins divide thus (Schaefer, 1938): *star-like expansion pattern*: ovalbumin, pepsin, pepsinogen, tobacco seed globulin, trypsinogen, urease, edestin, and TMD virus; *rough circular expansion pattern*: trypsin, papain, and gliadin; *smooth circular expansion pattern*: insulin, zein, casein, protamine, and gelatin. The relation between these effects and the surface film's viscosity is as follows:—

	Viscosity (in the former units)
<i>Star-like expansion pattern</i>	
Pepsin.....	0.75
Ovalbumin.....	0.28
Tobacco seed globulin.....	0.20
<i>Rough circular expansion pattern</i>	
Trypsin.....	0.23
Papain.....	0.18
Gliadin.....	0.003
<i>Smooth circular expansion pattern</i>	
Insulin.....	0.028
Zein.....	0.003
Casein.....	0.010

³ Phenomena of this kind may sometimes be seen at the surface of a cup of tea where a film is first formed by tanned plant protein, and this is then split along sharp edges as the oil from the milk expands with greater force beside it.

From this list it is seen that if, as we must suppose, the expansion pattern is a delicate test for molecular elongation the surface film viscosity is not related to it in a simple manner. But it may be significant (although unfortunately we have not worked on many of the same proteins) that ovalbumin, which comes in our group B₂, has a high surface viscosity and a *star-like* expansion pattern, while insulin, which comes in our group C, has a low surface viscosity, and a *smooth circular* expansion pattern.

Schaefer (1938) further reports the extremely interesting fact that if pepsin is subjected to prolonged heat denaturation, its expansion pattern changes from *star-like* to *smooth circular*, suggesting that denaturation has formed elongated fibrils but that these have become clumped in loose tangles as if in a coagulum and can no longer contribute to any cohesion anisotropy of the surface film. This corresponds with our experience, for, as we have seen above, ovalbumin or amphibian embryo euglobulin, denatured by guanidine in the bulk phase, will not at neutral pH form the usual surface film showing anomalous flow. In general, denatured proteins spread on surface films more unwillingly than native ones, but if the pH is adjusted to the acid side, they will do so (Bull, 1938).

Böhm and Signer (1931) denatured ovalbumin with alkali (pH 13). Gel strain-birefringence made its optical examination difficult to interpret, but variable pressure capillary viscosimeter readings indicated highly elongated particles. After some hours, however, this anomalous viscosity quite disappeared, probably owing to tangle formation.

In general, when globular corpuscular proteins are denatured with strong urea solutions (Liu, 1933; Bull, 1940 for ovalbumin; Neurath and Saum, 1939; Neurath, Cooper, and Erickson, 1942*a* for serum albumin; Neurath, Cooper, and Erickson, 1942*b* for serum pseudoglobulin) or by heat (Anson and Mirsky, 1932 for ovalbumin; Loughlin and Lewis, 1932 for haemoglobin), the relative viscosity rises greatly but there is neither anomalous viscosity (measured in the capillary instrument under varying pressures) nor flow-birefringence at any stage. All this agrees with the view that tangles are produced from the fibrils into which the protein cage molecules are first transformed. There would also be a general increase of intermicellar forces.

But when fibrillar corpuscular proteins are denatured (Frampton, 1939, for tobacco mosaic virus nucleoprotein; Edsall and Mehl, 1940 for myosin; and experiments reported in the present paper) anomalous viscosity and flow-birefringence are abolished, and there is a fall of relative viscosity. Increase of intermicellar forces and tangle formation must therefore probably be over-compensated by loss of relative viscosity due to the decreased asymmetry of the particles.

These views somewhat resemble the earlier conceptions of the Peiping school, who greatly emphasised Chick and Martin's distinction (1911) between two stages of denaturation, denaturation proper being followed by coagulation or

agglutination, (Wu and Wu, 1925; Wu, 1927, 1929). Thus it has been stated by Wu and Chen (1929) that denaturation always brings about a large increase of acid- and base-binding power of a protein, but the quickly succeeding coagulation reverses it, ending in a final level less than that of the native protein.

Conversely, insulin, according to Schaefer (1938), if treated with copper or zinc, will give a *star-like* expansion pattern instead of a *smooth circular* one. This would suggest that it has been changed into something capable of forming rod-like or fibre-like particles. That this can easily happen to insulin has been shown by Waugh (1941). If a 2 per cent solution of insulin HCl be boiled for $\frac{1}{2}$ hour it forms a thixotropic gel, and this on dilution gives strongly flow-birefringent solutions, of high viscosity. By alternate freezing and thawing the flow-birefringence and viscosity disappear, and the protein resumes its normal properties. Hormone activity is retained throughout. This can hardly mean anything but reversible linear aggregation.

On denaturation, globular corpuscular proteins give fibre protein x-ray pictures (Astbury and Lomax, 1935); and the Langmuir-Schaefer expansion patterns make it extremely probable that the surface films of corpuscular proteins are composed of unrolled fibrils. Indeed, as long ago as 1882 von Ebner, forcing out ovalbumin solutions into absolute alcohol through a capillary pipette, obtained highly birefringent fibrils. But the evidence which clinches the matter is that of Astbury, Bell, Gorter, and van Ormondt (1938) and Stenhagen (1938), who, building up piles of protein monolayers on a chromium-plated metal slide mechanically moving repeatedly through a protein film at an air-water interface (the technique of Blodgett, 1935; Blodgett and Langmuir, 1937), were able thus to produce multilayers containing from 1400 to 1800 layers of ovalbumin film, each one 9 to 10 Å thick. Such multilayers, stripped from the metal base, were found to be birefringent (as much so as wool), while within them were often contained a multitude of negative tactoids, probably caused by the presence of minute foreign bodies, and showing at their edges an intense birefringence (as high as that of natural silk). Examination by x-rays confirms the presence of innumerable extended polypeptide chains lying roughly parallel to the direction of movement of the slide, with their side-chains roughly perpendicular to the plane of the film. These chains must have pre-existed in the monolayer at the air-water surface, and in forming them the ovalbumin corpuscular cage molecules must therefore have unfolded or uncoiled. Further proof of the fibrous nature of these films was their tendency to tear parallel to the direction in which the slide was moved through the air-water interface, and in some cases they even showed a fringe of delicate fibres along their edges at right angles to this direction. These interpretations are strikingly confirmed by the anomalous viscosities of surface films described by us.

It may be mentioned here that our surface films of proteins are not the first for which anomalous viscosity has been described. Fourn and Harkins (1938)

have given anomalous flow diagrams closely analogous to ours for condensed monolayers of the long-chain alcohols (C14-C18). This non-Newtonian flow only occurs above a certain kink-point as pressure on the monolayer is increased.

In the absence of flow-birefringence observations on the protein surface films, and apart from all other evidence, our measurements of their anomalous viscosity cannot indeed be said to prove the existence of fibrillar particles in them. The anomaly might be a genuine "structural viscosity" due to the existence of intermicellar forces between disc edges, which increasing shear force would gradually overcome. But the occurrence of bulk anomalous flow after the dispersion of the film, makes this point of view difficult to hold, and it would also be hard to believe that in those cases where normal flow is obtained in the film no such intermicellar forces exist.

The work of the past 20 years has familiarised us with the conception of protein denaturation as essentially the transformation of compact, rigid, almost crystalline, coiled or folded polypeptide-chain molecules with a highly specific configuration, maintained by secondary valencies, hydrogen bonds, sulphydryl linkages, etc.; into unrolled, more flexible, fibrillar molecules with a less specific configuration. Obviously this might involve profound chemical changes in the molecules. Of several papers outstanding from the mass of literature, the pioneer work of Wu (1931) and Mirsky and Pauling's continuation of it (1936) and the work of Astbury, Dickinson, and Bailey (1935) may be mentioned here. The decrease in solubility on denaturation is explained by the assumption that in the native molecule the polar groups are mostly on the external surface of the framework, but when the molecule unrolls, the non-polar hydrophobe groups are bared. The appearance of -SH groups is explained by their being no longer employed in cross-chain linkages. The crystallisation of native proteins and the incapacity of denatured proteins to crystallise is explained by the view that they are no longer rigid and compact structures. On the other hand the denatured fibrils may participate in forming regular submicroscopic bundles, or liquid crystals, and it is possible that the behaviour of the surface films described in the present paper should be interpreted from this point of view (*cf.* Lawrence, 1938). In the modern view, all the diverse methods of denaturation can be understood. Drying withdraws water molecules from the framework causing it under some conditions to crumble; heat and radiant energy shatter the secondary valency bonds; shaking and gas bubbling uncoil the frameworks at the air-water interfaces; urea and guanidine derivative molecules penetrate into the frameworks and disrupt them; organic solvent molecules also penetrate and by dissolving the non-polar groups, turn the frameworks inside out, and so on. The denaturation process may be up to a certain stage reversible, as Anson and Mirsky (1925, 1931) and Wu and Lin (1927) were the first to point out. Little, of course, is as yet known about

the actual structure of the framework; if the geometrical formulations of Wrinch (1937), which have aroused so much interest, are untenable, some other such formulations will have to be proposed. The framework conception does, however, enable us to understand how isotopic amino acids can so quickly interchange with the amino acids in the protein molecule (*cf.* Rittenberg, 1941), for if the main chain were not coiled and held together by many secondary valencies and similar bonds, the peptide linkages in the main chain could hardly open and close as frequently as it seems they do.

According to the view adopted here, it may be a great mistake to suppose that the uncoiled protein chains have no physiological significance. As was indicated in the introduction, the assumption of a "dynamic framework or lattice" (*cf.* Needham, 1942, page 658; Schmitt, 1939) of fibre molecules in the cell fills a real need in causal morphology, experimental embryology, histology, and the like; and there are numerous facts, especially concerned with the existence of liquid crystalline phases in the cell, which support it. Most germane to the present paper is the direct proof which Pfeiffer (1937) has given that fibril molecules are contained in living protoplasm. If naked cytoplasm of animal eggs (molluscs, echinoderms) or of plant cells (such as the liquefying pericarps of *Solanum* or the parenchyma cells of hyacinths and orchids) is caused to flow through a minute capillary viscosimeter under different pressures, curves exactly analogous to those reported in the present paper, showing marked anomalous viscosity, are obtained. Moreover, we have evidence that such forced flow of protoplasm may have important biological consequences; thus P. E. Lindahl found that if echinoderm eggs are squeezed through a capillary tube, the leading end of the "sausage" always afterwards becomes the ventral pole.

The question of the biological status of proteins in the denatured condition is important. When Banga and Szent-Györgyi (1940) find flow-birefringence in urea-treated tissue globulin solutions; when we, as here, find anomalous viscosity in protein surface films—are these phenomena of physiological significance or not? There is considerable reason to think that they are. We are not without evidence that an appreciable proportion of the protein in the living cell is in the denatured state.

If denaturation be taken as synonymous with the unrolling of a corpuscular protein, this must occur at all oil-water interfaces in the cell, *e.g.* at the surfaces of mitochondria, the cell membrane, the intracellular oil droplets, etc. The very low surface tension (< 1 dyne per cm.) found by Harvey and Shapiro (1934) and Harvey and Schoepfle (1939) for intracellular oil droplets was shown by Danielli and Harvey (1934) to be due to protein, and Danielli later (1938*a*) found that these low tensions are produced at a wide variety of oil-water interfaces by a wide variety of corpuscular proteins. There is little specificity. Lastly, Askew and Danielli (1936, 1940) proved that the protein absorbed at the oil-water interface is actually unrolled.

If denaturation be taken as synonymous with loss of solubility, there is direct evidence that forms of the same protein with different solubilities occur in the living cell. Many workers have established that in alkaline rigor, frog and rabbit myosin is less soluble than in resting muscle (Mirsky, 1938). The same thing occurs in limb muscle of crabs in iodoacetate rigor, or as a result of exercise to exhaustion (Danielli, 1938*b*). Mirsky, too, (1936) has shown that a certain protein fraction in sea urchin eggs becomes less soluble after fertilisation.

The question of the retention of specificity and biological activity by surface-denatured uncoiled protein is also relevant, and we now have a certain amount of information on it. It is true that the differences between haemoglobins of different animal species (crystal form, solubility, gas affinities, position of absorption bands, etc.) disappear on denaturation to their haemochromogens by acid and alkali (Anson and Mirsky, 1925); that denatured proteins lose immunological specificity (Zinsser and Ostenberg, 1914; Wu, TenBroeck, and Li, 1927) and that pepsin and trypsin denatured irreversibly with heat or acids can perform proteolysis no more (Northrop, 1930, 1932). But these transformations were not carried out by surface film unrolling. It may indeed be that it is only upon tangle formation (see foregoing) that biological activities and specifications are finally lost. Pepsin has been obtained by Gorter (1937) and Langmuir and Schaefer (1939) in the unfolded surface film state—it retained its activity. Catalase retains in the film 20 per cent of the activity it possessed when in solution (Langmuir and Schaefer, 1938; Harkins, Fourn, and Fourn, 1940), and though urease is apparently inactivated (Langmuir and Schaefer, 1938), saccharase is not (Sobotka and Bloch, 1941). Rothen, Chow, Greep, and van Dyke (1941) studied insulin and certain pituitary hormones. The activity of insulin they found to be unimpaired by surface denaturation, but that of the posterior pituitary oxytocic pressor hormone and that of the pituitary gonadotrophic hormone were markedly reduced or destroyed. On the other hand, the latter protein hormone had in no way lost its capacity to combine with homologous antibodies. Again, though surface films of *Pneumococcus* antibody cannot combine with their specific polysaccharide (Danielli, Danielli, and Marrack, 1938) avian ovalbumin in the surface film does not lose its property of combining specifically with antibodies of homologous sera (Rothen and Landsteiner, 1939) and a streptococcal agglutinin nucleoprotein remains immunologically active in the monolayer (Chambers, 1939). Finally, myosin shows adenosine triphosphatase activity, not only in the bulk phase, but also when partially dried and in visible fibre form (Engelhardt, Ljubimova, and Meitina, 1941). One may conclude that although some biological specificity and activity may be lost when the protein cages unroll, by no means all of it disappears until the unrolled fibres finally intertwine chaotically.

If then we may adopt the hypothesis that protein molecules unrolled into elongated fibrils by denaturation have a part to play in the dynamic architecture of the cell, as well as those which are elongated before denaturation, the question arises as to how they are produced in the living organism? Is it possible that the rotational churning and streaming motion of protoplasm has some connec-

tion with this? The streaming of protoplasm has often been considered only in connection with amoeboid movement, but the classical cyclosis of the cytoplasm in plant cells and the extraordinary churning movements in eggs, especially before cell division (see, for example, the descriptions of Harris, 1935) cannot have this significance. It is worth asking whether they might not play a part in the orienting of surface-denatured fibrils by shear forces into those anisometric micelles which the cell shape itself (as in the neural plate of the embryo), or the egg's polarity and symmetry properties, or the different times of determination of limb bud spatial axes, seem all to imply. It may be said that the speed of cytoplasmic streaming is low, and hence the shear forces associated with it would be much lower than those which we have in our instruments, but this difference is offset by the probability that on the other hand the effective near-molecular annulus equivalent in which the shear forces would act in the cell would be enormously smaller than in our instruments, so that the actual shear rate might, on balance, be very much higher. The spinning of long protein molecules by protoplasmic streaming is a conception analogous to that which Preston (1941) has invoked to account for the "crossed fibrillar" structure of cellulose chains in the walls of plant cells; he supposes that it derives from alternate contractions and relaxations of protein chains in the cytoplasm. This affords another instance of the universally felt need for chain structures in cells.

Lastly, if the above argument is sound, it is not without interest that in modern human technology, the preparation of actual fibres such as the artificial wool (lanital) from casein (von Weimarn, 1927; Sutermeister and Browne, 1939, p. 215), plant seed globulins such as edestin and excelsin (Astbury, Dickinson, and Bailey, 1935; Labarre and Dostert, 1942), and soya bean protein (Satô, 1923; Horvath, 1938) would be an instance of man having unknowingly made use of certain properties of proteins which have been, since the beginning of living things, essential to the life and development of cells and organisms.

SUMMARY

1. An extensive investigation has been made of protein particle shape using the methods of flow-birefringence and anomalous viscosity measurement in the coaxial cell.

2. As a result of investigations on a number of proteins, it is concluded that they may be divided into four groups.

Group A consists of those which show flow-anomaly both in the bulk phase and in the surface film. These also show flow-birefringence in the bulk phase. Examples: tobacco mosaic disease virus nucleoprotein; myosin. Though corpuscular proteins, they have elongated particles before denaturation.

Group B consists of those which show flow-anomaly only (in the first instance) in the surface film, and no flow-birefringence in the bulk phase. They are

probably close to spherical in shape in solution, but form elongated particles as they denature in the surface film. After this process has been completed, they may show flow-anomaly also in the bulk phase. Some proteins show flow-anomaly in the surface film immediately it forms, others only show it after a certain time has elapsed for the building up of the film. We designate the former as group B₁ and the latter as group B₂.

Group B₁, immediate surface film flow-anomaly. Examples: serum euglobulin, amphibian embryo euglobulin *b*.

Group B₂, slowly appearing surface film flow-anomaly. After the film has once been fully formed and then dispersed by shaking, the solution may have the properties of that of a protein in group B₁; *i.e.*, anomalous flow in the film may occur immediately on testing in the viscosimeter. Examples: avian ovalbumin, amphibian embryo pseudoglobulin.

Group C consists of those proteins which show flow-anomaly neither in the bulk phase nor in the surface film, under the conditions used by us. They are probably close to spherical in shape. Examples: insulin, methaemoglobin, amphibian embryo euglobulin *c*, mucoproteins.

3. The theoretical significance of protein fibre molecules, whether native or formed by denaturation in the living cell, is discussed, especially in relation to experimental morphology and cytology.

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THE OSMOTIC BEHAVIOR OF CRENATED RED CELLS

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A survey of the results of the experiments which have been done on the volume changes of mammalian red cells in solutions of various tonicities shows that the red cell sometimes behaves as a perfect osmometer, while at other times it does not (Ponder, 1940). More specifically, if a red cell, the initial volume of which is denoted by 100, is immersed in an infinite volume of a medium of tonicity T , the new volume which the cell ought to take up by the exchange of water alone is

$$V = W(1/T - 1) + 100 \quad (1)$$

where W is the percentage of water in the cell by volume, and where T is the depression of freezing point of the suspension medium divided by the depression of freezing point of the undiluted plasma. When V is measured experimentally and compared with V in expression (1) it is often found that there is a considerable discrepancy, the swelling of the cell being described by the expression

$$V = RW(1/T - 1) + 100 \quad (2)$$

where R is a "correction factor introduced to reconcile observation with theory."

Three suggestions have been put forward as to the meaning of the constant R , and two of these have already had to be abandoned, at least as complete explanations. The first is that R measures the fraction of cell water which is free, the remainder being bound. To account for the variations in the value of R , this explanation requires that the amount of free water varies, in an unexplained way, from about 50 per cent of the total cell water to about 100 per cent, quite apart from its being very doubtful that the red cell contains any appreciable quantity of bound water at all (Hill, 1930; Macleod and Ponder, 1936). The second explanation is that the cell reaches osmotic equilibrium by losing or gaining osmotically active substances as well as by gaining or losing water; under these circumstances the percentage of osmotically active substance lost or gained would be $100(1 - R)(1 - T)$. The quantity of salt lost from red cells into hypotonic solutions, however, is much smaller than this explanation demands (Ponder and Robinson, 1934; Davson, 1934; Ponder, 1940). The third explanation is one to which I called attention in 1940, and is that the entry of water from hypotonic solutions, and the loss of water to

hypertonic solutions, is opposed by elastic forces of which R is an indirect measure. It is with this possibility that this paper is concerned.¹

I. Shape Changes and Elastic Forces

It is customary to describe the swelling of red cells in hypotonic solutions as taking place by the biconcavities turning inside out, the short axis of the cell increasing while the diameter decreases, and the cell ultimately hemolyzing either as a spheroid of low eccentricity or as a sphere. This picture of what happens is all very well for the purposes of mathematical (Ponder, 1924) or illustrative (Haden, 1934) treatment, but in the case of the individual red cell it does not represent the facts. So far as individual cells are concerned, the shapes assumed during swelling are very variable and cannot be accounted for on any supposition other than that they have rigidity of form.

(a) If the red cells of man or of rabbit are examined in hypotonic media in which the extent of the swelling of the average cell is known (*e.g.*, in hypotonic plasma of $T = 0.7$, in which the volume is about 130 per cent of the volume in undiluted plasma), the swelling will be found to be accompanied by different shape changes in the case of different cells. Some cells retain their biconcave form, apparently increasing in volume by increasing their thickness uniformly or by becoming a little thicker at the ends. Others become cup-shaped, one biconcavity being obliterated. Still others are spheroidal and convex, and a few are spheres. If the cells show crenation in the isotonic medium, the crenation may persist in the hypotonic medium, and swollen cells may present it to a varying degree. Neighboring cells do not necessarily swell to the same extent; and a crenated thorn-apple form may be seen side by side with a spheroidal or a cup-shaped cell which is obviously swollen. The most superficial observation shows that the individual cell does not always behave as if it were a balloon surrounded by a uniform non-elastic membrane, for the radii of curvature for different points on the surface assume such a variety of values that it is necessary to suppose either that the tensions vary enormously, *i.e.*, that there is non-uniformity of structure and rigidity of form, or that the cell is sometimes not a balloon-like body, but a gel.

(b) As swelling proceeds, the diameter of the average cell decreases about 8 per cent as the thickness increases. This observation (Ponder, 1924, 1933) has been confirmed by Teitel-Bernard (1932) and by Haden (1934). The

¹ A fourth explanation, that the values of R which are significantly less than 0.95 (this would allow 5 per cent of the total cell water to be "bound" as water of hydration or hemoglobin) are due to experimental error, is quite untenable. Even when one makes every allowance for the errors which may occur in the measurement of red cell volume, one cannot explain away the frequency with which low values of R have been found by different observers, working independently, and using a variety of methods.

decrease in diameter is what would be expected of a spheroidal balloon-like body surrounded by a membrane and containing fluid contents, but the proof that a decrease in diameter would occur in such a body during swelling (Ponder, 1924) depends absolutely on the membrane having elastic constants.

(c) If we trace the changes in shape and area which occur as swelling proceeds, we get a diagram which is incompatible with anything except a body with rigidity of form. Let us represent increase in volume on the abscissa of Fig. 1,

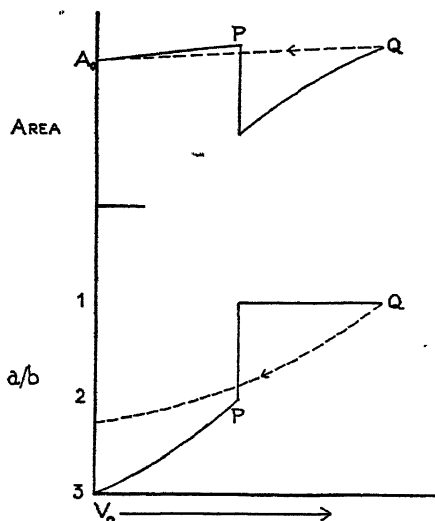


FIG. 1. Upper ordinate, red cell area, with initial area A_0 ; lower ordinate, length/thickness ratio a/b . Abscissa, red cell volume, with initial volume V_0 . Solid lines, course of events as cell swells; dotted lines, probable course of events after lysis. P , yield point; Q , critical volume. The cell is represented as returning to its critical volume but not to its original a/b ratio because it does not usually return completely to the original biconcave discoidal form (see Furchgott, 1940).

starting with the initial volume V_0 , and let us also represent the cell area, starting with the initial area A_0 , and the length/thickness ratio a/b as a rough measure of shape,² on ordinates. (1) As swelling proceeds, the area of the cell increases only slightly, and the increasing volume is accommodated by a change in shape, so that the length/thickness ratio becomes smaller. The constancy of the area must mean that the extensibility of the membrane is not very great. (2) As the swelling continues, a point is reached (the point P in the diagram) at which some structure appears to give way and at which the cell becomes a

² The use of the length/thickness ratio as a measure of shape again involves the introduction of an abstraction which may have little meaning in the case of an individual cell (see (a) above).

sphere. With this sudden change in shape the a/b ratio becomes 1.0 and the area decreases. The yield point P may correspond to smaller volume increases in the case of some cells than in the case of others. (3) With further increases in volume, the area of the sphere increases until hemolysis occurs when the critical volume is reached at the point Q , the ratio a/b remaining unity. The critical volume may be smaller under some circumstances than under others, *e.g.*, when the cells are suspended in saline instead of in plasma.

This diagram clearly applies to a body which has rigidity of form. If we replace the units of volume on the abscissa with units of force, it becomes a stress-strain diagram, and the line V_0P represents the behavior of the body within the elastic limits of its materials. We can certainly make this change in the units on the abscissa in cases in which the constant R of expression (2) is found experimentally to be less than unity, because under such circumstances we have experimental justification for thinking that an elastic force opposes osmotic swelling. It will be shown below that R is less than unity under circumstances which are associated with crenation and gelation, when the elastic resistance to osmotic swelling is presumably great. When R is found experimentally to be unity, on the other hand, the elastic resistance offered by the cell material must be too small to measure.³

(*d*) When the critical volume is reached the cell hemolyzes and the stretching forces disappear. The hemolyzed ghost then returns more or less completely to the shape of the cell from which it was derived (dotted lines in Fig. 1), and this in itself is evidence that its materials possess elastic properties, particularly as the return to the discoidal, or at least spheroidal, form takes only "a fraction of a second" (Teitel-Bernard, 1932).

II. Crenation and Gelation

The changes in red cell shape during swelling suggest that its materials possess rigidity, but do not decide the question as to whether the rigidity is sufficient to resist osmotic swelling to a measurable extent. About fifteen years ago, when the tendency was to look upon the red cell as a body with fluid contents and possessing no structure except for a membrane about 100 Å thick, it was difficult to think of it as a structure sufficiently rigid to oppose

³ It should be noticed in this connection that the R values of experiment are nearly always less than 1.0, and rarely greater. This makes it likely that the small downward deviations of R have a real significance, and are not due simply to experimental error. We often, for example, find an R value of 0.9, and, adding up all the possibilities of experimental error, we have to admit that an error of 0.1 is possible. But we rarely find an R value of 1.1. The fact that so many of the high values turn out to be a little less than 1.0 can be accounted for by supposing that only some 95 per cent, instead of all, of the cell water is "free," but until we know exactly what the figure for the "free" water is, there is the possibility that even values of R in excess of 0.9 are indirect measures of a small elastic resistance.

osmotic forces of the order of 1 atmosphere. We now know more about red cell structure and about the influence which the interior exerts on shape transformations (Ponder, 1942), and if we consider that under some circumstances the red cell interior may actually be a gel, the existence of elastic forces sufficient to resist osmotic swelling becomes not only possible but probable. Since it is in the case of crenated cells that the evidence for rigidity and the existence of forces opposing osmotic swelling is the strongest (section I, *a*, and section III), we shall advance the argument considerably if we are able to show that crenation and gelation go hand in hand.

The likelihood of their doing so will appear greater if we consider how little water has to be abstracted from the cell in order to convert its interior into a gel. Although it can now be regarded as settled (Waller, 1935; Ponder and Saslow, 1930b) that crenation does not necessarily correspond to a diminution in cell volume, it nevertheless tends to occur under conditions in which water is abstracted, *e.g.*, in hypertonic media and after the addition of oxalates. Since the molecular weight of the "wet" hemoglobin crystal (hemoglobin + water) is 132,000 and that of the hemoglobin molecule alone is 66,700, each 66,700 parts of hemoglobin contains 65,300 parts of water. The "wet" crystal therefore contains about 49 per cent of water; the red cell itself, however, contains about 60 to 65 per cent of water, and so by the abstraction of a comparatively small quantity, such as certainly could be removed either by hypertonic solutions or by the addition of an excess of oxalate, its interior will be brought into the state of the wet crystal, which is a rigid structure.

Teitel-Bernard (1932), indeed, regards crenation as one stage in the formation of intracellular crystalline hemoglobin. He observes that crenated red cells show more birefringence than normal cells do, and attributes this to an increasing degree of orientation of the hemoglobin which ultimately leads to intracellular crystal formation. This takes place in several stages, each more stable than the one before it. In the first stage, which corresponds to the reversibly crenated erythrocyte, local areas of gelation appear, and in these areas, which correspond to the individual crenations, the hemoglobin molecules are lined up along their axes of crystallization. The orientation is reversible at first, and the crenations can be made to disappear by mechanical, chemical, or osmotic means. In the more advanced stages the gel formation and the orientation of the hemoglobin are more permanent, and in the final stage crystals of hemoglobin are seen. Teitel-Bernard thinks that the gel which is formed in the earlier stages is actually a hemoglobin gel formed as a result of the destruction of an equilibrium of some kind, as by the withdrawal of water or the changes of pH towards 6.5, the isoelectric point of hemoglobin. It confers on the crenated cells a mechanical rigidity which can be demonstrated by microdissection methods, and they are sometimes so completely gelled that they do not lose their contents even when torn by the microdissection

needle.⁴ The conclusion that the crenated red cell has an internal skeleton which gives it mechanical rigidity is in keeping with a number of other observations in the older literature, such as the observation of Schafer that red cells can sometimes be cut in two without losing their contents and the description by Rockwood of red cells which are penetrated by glass particles without their hemoglobin escaping.

Generally speaking, the literature treats the phenomena of crenation in a perfunctory fashion, and does not make sufficient distinction between one form of crenation and another. There seem to be at least three forms. (1) One kind of crenation is often associated with the abstraction of water from the cell and with a diminution in cell volume, although diminution in volume is not necessarily accompanied by crenation. This type occurs when the cells are exposed to hypertonic solutions, or when oxalates, fluorides, etc., are added to plasma as anticoagulants. There is no difficulty in seeing how it could be the result of gelation of hemoglobin and perhaps also of whatever other proteins exist in the cell interior. (2) Human cells in saline media containing acetate buffers begin to crenate at $\text{pH} = 7.0$ when the pH is reduced towards 6.5, the isoelectric point of hemoglobin, and the crenations persist until about $\text{pH} 6.0$, below which the cells are discoidal until they begin to hemolyze at about $\text{pH} = 5.4$ (Claudio, 1931; Teitel-Bernard, 1932). This type of crenation appears to be associated with the intracellular crystallization of hemoglobin, no doubt preceded by gel formation. (3) Washed human red cells in saline media become crenated if the pH is increased above 8.0, and assume the perfectly smooth spherical form at about $\text{pH} = 9.2$. The appearance of crenation and the assumption of the spherical form, at first crenated and later smooth, can be prevented between $\text{pH} 8.0$ and $\text{pH} 10.0$ by the presence of the antisphering substance of plasma, shown to be an albumin by Furchgott (1940) and Furchgott and Ponder (1940). This type of crenation is probably due to an inability of the red cell membrane to maintain its special shape rather than to a process of gelation.

⁴The changes observed when a crenated cell is pricked with the microdissection needle are different from those which follow the pricking of a normal cell. The normal cell becomes spherical, fades, and becomes a ghost, which momentarily reassumes the flattened shape of the original cell before shrivelling up into a viscous mass on the end of the needle. Some crenated cells behave similarly, but fade more slowly, as if their contents were more viscous. Others become spheres, but do not lose their contents, and some can be transfixed without hemolyzing. They seem to be irreversibly gelated (Teitel-Bernard, 1932).

No direct measurements of tension and elasticity similar to those of Cole (1932) for the *Arbacia* egg or of Norris (1939) for nucleated erythrocytes exist for the crenated mammalian red cell. It is not unlikely that the values for the normal mammalian red cell would be small, like Norris' values, but that those for crenated cells would be very much greater.

The pH range in which crenation is absent in saline media is therefore small (pH 7.0 — 8.0), and it is only when blood is heparinized, hirudinized, or defibrinated that we can expect crenation to be absent in plasma. Even under these circumstances crenation may occur for reasons not yet understood and apparently peculiar to the individual, and when it occurs it may affect only a few of the red cells, or many.⁵

III. Crenation and R Values

Proceeding on the assumption that the appearance of crenation is a sign of gelation in the red cell interior, we may enquire whether the values of R are smaller when red cells are crenated than when they are not. To do this we require to measure the swelling of cells in plasma of known degrees of hypotonicity, and, while this may be done by a variety of methods, the procedure described by Shohl and Hunter (1941) is the most satisfactory. Their method is a colorimetric one which differs from the original method of Stewart (1899), and from Ponder and Saslow's modification of it (1930a), in that the dye Evans blue (T-1824) is used in place of a solution of hemoglobin. The method is used in the following way for obtaining R values in hypotonic plasma.

Evans blue (Eastman Kodak) is dissolved in distilled water in a concentration of 600 mg. of dye per liter. Exactly 0.5 ml. of the solution is placed in a series of tubes (100 mm. by 13 mm.) and is evaporated to dryness at 70°C. It is convenient to prepare a few dozen tubes at a time, and the success of the method depends on the accuracy with which the same volume of the dye solution is delivered into each. Blood is obtained in the usual way with either heparin or oxalate as the anticoagulant, and about 1.5 ml. is centrifuged to give plasma, of which 0.5 ml. is added to one of the tubes containing dye. The tube is shaken to dissolve the dye, and 0.1 ml. of the dye-stained plasma is transferred to a tube containing 10 ml. of saline (tube P_0). To another tube (tube O_0) containing 10 ml. of saline is added 0.1 ml. of the plasma itself. One ml. of whole blood is now placed in a tube containing dye, which is dissolved by stoppering the tube and shaking gently. The tube with its contents is then gently centrifuged, and 0.1 ml. of the supernatant dye-stained plasma is transferred to another tube containing 10 ml. of saline (tube B_0). Using the contents of tube O_0 for setting the zero, opacity readings for the contents of tube P_0 and B_0 are made with a lumetron photometer; the amount of plasma, in milliliters, contained in the 1 ml. of blood is then obtained by dividing the reading P_0 by the reading B_0 .

⁵ Guest and Wing (1942) have recently described an ingenious variety of hematocrit method by means of which the swelling of red cells can be followed even in solutions so hypotonic as to produce lysis. They find that normal human red cells (heparinized blood) tend to swell as perfect osmometers. Dr. Guest writes me, however, to say that the red cells in sickle cell anemia and Mediterranean anemia (Cooley) swell less than would be expected of a perfect osmometer in hypotonic saline. The interest attached to this observation, so far as this paper is concerned, lies in the close relation between sickling and extreme forms of crenation.

and multiplying by 0.5. The volume V_0 occupied by the cells is obtained by subtraction.

Two ml. of whole blood is next placed in a test tube and centrifuged gently so as to produce a layer of clear supernatant plasma. A volume of water equal to the volume of plasma known to be contained in the 2 ml. of blood, or $2(1 - V_0)$ ml., is added in such a way as to form a layer over the supernatant plasma. The tube is then stoppered and the contents are rapidly mixed by shaking. One ml. of the mixture is added to a tube containing dye, and after the dye is dissolved 0.1 ml. is transferred to a tube containing 10 ml. of saline (tube B_1). The rest of the mixture is centrifuged to give diluted plasma, of which 0.1 ml. is added to 10 ml. of saline (Tube O_1) and 0.5 ml. to a tube containing dye. When this is dissolved, 0.1 ml. of the dye-stained diluted plasma is transferred to 10 ml. of saline (tube P_1). The photometer zero is set for the contents of tube O_1 , and $0.5 P_1/B_1$ gives V_1 , the new volume occupied by the swollen cells, the number of which, however, has been reduced by the addition of water to the whole blood. The percentage swelling, accordingly, is

$$V_{\text{exp.}} = \frac{100V_1}{V_0 \cdot \frac{2}{2 + 2(1 - V_0)}} \quad (3)$$

$$= \frac{100V_1(2 - V_0)}{V_0} \quad (4)$$

which is to be compared with the swelling of a perfect osmometer in a medium of tonicity T , in these experiments 0.5

$$V_{\text{calc.}} = W \cdot \frac{\phi - \phi T}{\phi T + 1} + 100 \quad (5)$$

in which W is the quantity of water contained in the cell and expressed as a percentage by volume, and ϕ the ratio of the volume of the hypotonic medium to the volume of the cell water. R , the constant under discussion, is equal to $(V_{\text{exp.}} - 100)/(V_{\text{calc.}} - 100)$.

This method is far more satisfactory than any other method for measuring red cell volume that I have used, and its accuracy seems to be limited only by the precision with which photometric readings and delivery from pipettes can be made.⁶ It has all the advantages claimed for Ponder and Saslow's modification of Stewart's colorimetric method, with the additional advantage that dye is used instead of a hemoglobin solution, and so the risk of error due to lysis of a few of the cells of the system is minimized. I have compared the results given by Shohl and Hunter's method with those given by the hematocrit, and

⁶ An important technical point is that the transfer of dye-stained plasma is carried out with the special pipettes described by Levy (1936), from which very accurate delivery can be made. For measuring out the dye, I use 0.5 ml. micro pipettes ("to contain") with a ground tip and a constriction in the region of the 0.5 ml. mark.

have obtained good agreement within the limits of error to which the hematocrit method is subject. In previous investigations on the swelling of red cells in hypotonic media there has always been some question as to the reliability of the method used (speed in the case of the hematocrit, the value of the form factor in the case of conductivity measurements, the wave length, etc., in the diffractometric methods, the possibility of hemoglobin escaping from red cells in the original colorimetric method, and so on), but the results obtained by this colorimetric method are not open to question on any such grounds.

TABLE I

Experiment	Anticoagulant	R	Condition of cells
1	Heparin	0.97	No crenation
2	Heparin	0.95	No crenation
3	Heparin	0.93	No crenation
4	Heparin	0.93	No crenation
5	Oxalate	0.97	No crenation either in $T = 1.0$ or $T = 0.5$
6	Oxalate	0.96	Slight crenation in $T = 1.0$; no crenation in $T = 0.5$
7	Oxalate	0.89	Slight crenation in both $T = 1.0$ and $T = 0.5$
8	Oxalate	0.85	Slight crenation in both $T = 1.0$ and $T = 0.5$
9	Oxalate	0.75	Some crenation in $T = 1.0$; considerably less in $T = 0.5$
10	Oxalate	0.75	Some crenation in $T = 1.0$; a lot in $T = 0.5$
11	Oxalate	0.72	Every second cell crenated in $T = 0.5$
12	Oxalate	0.70	Like No. 11
13	Oxalate	0.69	Like No. 11
14	Oxalate	0.67	Like No. 11
15	Oxalate	0.59	Marked crenation in both $T = 1.0$ and $T = 0.5$
16	Oxalate	0.52	Marked crenation in both $T = 1.0$ and $T = 0.5$

This method was used to measure the swelling of human red cells from (a) heparinized blood (5 mg. heparin to 5 ml. of blood) and (b) oxalated blood (20 mg. sodium oxalate to 5 ml. of blood).⁷ The degree of hypotonicity established was $T = 0.5$ throughout. The cells were examined microscopically both in the undiluted plasma and in the hypotonic plasma, the purpose of the examination being to see if crenation was present, and if so, to what extent. The results of sixteen experiments are shown in Table I.

⁷ The quantity of sodium oxalate usually used as an anticoagulant is 10 mg. per 5 ml. of blood. In these experiments the quantity has been doubled because it is desired to exaggerate the effect. The amount of crenation produced, however, bears no simple relation to the amount of oxalate used. Factors of both time and temperature are involved, and very marked differences can be observed among the cells of different individuals. Crenation sometimes occurs in both heparinized and defibrinated blood.

It will be clear that the values of R in Table I depend in a general sort of way on the amount of crenation observed. In heparinized human blood crenation is usually absent, and R values found are usually high (0.9 or more). In oxalated blood, on the other hand, crenation is very often present, although its extent seems to vary from individual to individual for reasons still unknown. In any individual case, it should be observed that all the cells are not necessarily crenated to the same extent, and that some of them may appear normally discoidal while others are crenated; in some cases, moreover, crenation persists even in hypotonic plasma, while in others it is still in its reversible state and disappears as the cells swell. The lowest values of R are found when crenation persists in the hypotonic medium.

In estimating the amount of crenation in a preparation, one is helped by the fact that normal discoidal cells tend to form rouleaux, even in hypotonic plasma, whereas crenated ones do not. The latter therefore tend to lie apart from the rouleaux formed by the former. This is reflected in the observation that sedimentation is usually slower in oxalated blood than in blood which has been heparinized.

IV. Calculation of the Bulk Modulus

Suppose that the red cell swells to a volume $V_{\text{exp.}}$ when placed in a hypotonic solution of tonicity T , instead of to the larger volume $V_{\text{calc.}}$ given by expression (5). There must be some other tonicity T_1 , higher than T , in which a cell with an R value of 1.0 would be in equilibrium when it had attained the volume $V_{\text{exp.}}$, and this tonicity can be shown to be

$$T_1 = \frac{pW - (V_{\text{exp.}} - 100)}{p(V_{\text{exp.}} - 100 + W)} \quad (6)$$

which is the same as expression (1) when p is indefinitely great. Now T and T_1 could be converted into their corresponding osmotic pressures by multiplying by a suitable constant k , the value of which is about 7.5. The bulk modulus of the cell material, considered as a uniform gel, is therefore given by

$$K = \frac{k(T_1 - T)}{0.01(V_{\text{exp.}} - 100)} \quad (7)$$

which, to give K in the usual units of dynes/cm.², must be multiplied by 10⁶.

Taking the result of Experiment 16 in Table I as an example, we have $T = 0.5$, $p = 6.4$ (the initial volume concentration being 0.40), $V_{\text{calc.}} = 146$, and $V_{\text{exp.}} = 124$. This gives $R \approx 0.52$. Substituting in expression (6), we get $T = 0.664$, and substituting this in (7) gives $K = 5.1$ atmospheres or 5.1 (10⁶) dynes/cm.². This is the highest value found in this series of experiments, whereas the lowest value is zero if we suppose 5 per cent of the cell water to be bound to hemoglobin. The values of K are accordingly of the same magnitude as those found for gelatin gels (Northrop, 1927).

The variation in R between zero and about 0.5 in a series of experiments

which show crenation to an increasing extent may be due either to there being different degrees of crenation each associated with a different degree of rigidity and value of K , or to some of the cells being crenated while others are not. The value of K as measured is a mean value equal to $\Sigma nK/n$, and these experiments do not distinguish between the effect of different degrees of rigidity and that of the involvement of different numbers of cells. The second factor, however, clearly enters into such experiments as Nos. 11 to 14.

DISCUSSION

In surveying the many experiments which have been done on the changes in volume of the mammalian red cell in solutions of different tonicity, the result of one type of experiment has always been very difficult to explain. This is the observation that when swelling is measured in hypotonic plasma the value of R is usually much more nearly unity when the blood is defibrinated or heparinized than when it is oxalated (Ponder and Robinson, 1934). The difficulty has been the greater because in this type of experiment the possibilities of error are reduced to a minimum, and there is no doubt about the tonicity of either the plasma bathing the cells initially or the plasma which is rendered hypotonic. The anomalously small degree of swelling in oxalated blood is now satisfactorily accounted for by the observation that the cells are usually crenated, and by the demonstration that such cells swell as if they were elastic bodies with a bulk modulus of the same order as that for gelatin gels.

Whether the effects of crenation and gelation explain every low value of R which has been described is another matter. In many experiments in saline media and in media containing non-electrolytes, crenation must have been present without having been commented on. This is certainly true of many of my own experiments done between 1931 and 1940. Since crenation is part of the process by means of which the spherical form is produced, a certain amount of suspicion may now attach itself to those experiments in which diffractometric measurements of volume were made after the cells were converted into spheres between slide and coverglass (Ponder and Saslow, 1931), or by the addition of lecithin (Ponder and Robinson, 1934; Ponder, 1935). This suspicion, however, is probably not justified, for the type of crenation which occurs during the disk-sphere transformations seems to be distinct from that which occurs when water is abstracted from the cell or when the isoelectric point of hemoglobin is approached, and does not seem to be associated with gel formation.

In certain systems, the value of R may be less than that expected of a perfect osmometer for reasons other than the appearance of crenation with its associated elastic forces. In hypotonic glucose, for example, there is some loss of osmotically active substances in the case of the red cells of some species (Davson, 1934), and both Davson (1937) and I and my collaborators have found

some loss of potassium into hypotonic solutions and into hypotonic plasma. Although the losses have been too small to account for the values of R , it must be repeated with some insistence that they constitute presumptive evidence for the existence of a mechanism by which small amounts of osmotically active substances can pass across the red cell membrane.⁸ The swelling of ghosts in hypotonic media (Ponder, 1936) may be another example of a case in which more than a simple exchange of water is involved. While the problem of the anomalously small swelling of the red cell is greatly simplified by recognizing that crenation and gelation can result in the development of forces which oppose the osmotic forces, it cannot fairly be said that it is completely solved in the quantitative sense.

SUMMARY

The anomalously small swelling which the red cells of human oxalated blood undergo in hypotonic plasma is related to the extent to which the cells are crenated. Reasons are given for regarding crenation as corresponding to gelation, and the bulk modulus for crenated cells, calculated from the measurements of swelling in hypotonic plasma, is shown to be of the same order as that for gelatin gels.

ADDENDUM

A particularly stable form of gelation is observed when rat red cells are suspended in 3 per cent sodium citrate. Initially, the cells are discoidal or cup-shaped in this medium, and undergo the typical disk-sphere transformations between glass surfaces and on the addition of lecithin. When kept in the refrigerator (4°C.) for about 72 hours, the cells become irregularly crenated, and are apparently so gelled that they do not hemolyze even in distilled water, provided it is cold (10°C. or less). At higher temperatures, *e.g.*, 20°C., lysis occurs slowly, and at still higher temperatures, *e.g.*, 37°C., it is quite rapid; as the cells are left longer and longer in the refrigerator, hemolysis becomes progressively slower at all temperatures.

The gelled cells, while irregularly crenated, are essentially discoidal, but are unable to undergo disk-sphere transformations either between glass surfaces or when lecithin is added. The addition of 1 in 1000 saponin is followed by a slow hemolysis without the usual preliminary shape changes; the crenations do not disappear, nor does the cell become a prolytic sphere. In some cells one gets the impression that one part of the cell loses hemoglobin before other parts do, and the ghost, after lysis is complete, presents the same crenations as the original cell did. When the crenated cells are slowly warmed in the distilled water in which they have stood, most of them hemolyze without any change of shape, the pigment escaping and leaving a crenated ghost behind. A few cells, however, and usually those which are the most optically prominent

⁸ One can easily imagine a crenated, gelled cell losing electrolyte through an injured membrane without losing hemoglobin. In this way crenation might complicate experiments in which K loss is measured under different conditions.

in the field, lose their crenations, become prolytic spheres, and fade. Presumably these are the cells in which gelation is incomplete or still partly reversible.

This extreme resistance to hemolysis by water is observed when rat red cells are kept in the cold in 3 per cent and 4 per cent sodium citrate, but not when they are kept in 2 per cent citrate. It also occurs to a slight extent when the cells are kept at low temperatures in 1.5 per cent NaCl, and after several days some of the cells hemolyze only slowly when added to distilled water. When rat cells are kept in isotonic NaCl, NaCl to which oxalate has been added, or phosphate buffer at pH 6.5, hemolysis in water occurs as usual.

Human red cells kept at 4°C. for 5 days in the acetate buffer at pH 6.5 in which Teitel-Bernard finds crenation to be at a maximum, do not exhibit the extreme resistance to hypotonic hemolysis shown by rat red cells. Rabbit red cells resemble the cells of man rather than those of the rat.

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THEORY AND MEASUREMENT OF VISUAL MECHANISMS

X. MODIFICATIONS OF THE FLICKER RESPONSE CONTOUR, AND THE SIGNIFICANCE OF THE AVIAN PECTEN

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I

The retina of the zebra finch *Taeniopygia castinotis* (Gould) contains visual cells histologically of one general type only (cones), although some of these are differentiated by oil droplets; some are "double" cones; the retina has a well developed fovea. In keeping with the subsequently observed simplex character of the primary photoreceptor population, the flicker response contour (F vs. $\log I$), with white light and light-time cycle fraction $t_L = 0.50$, was found to be a single symmetrical probability integral (Crozier and Wolf, 1940-41 *b*). This was confirmed by a number of additional experiments with colored lights (Crozier and Wolf, 1941-42 *d*). The eye of *Taeniopygia* contains, however, a fairly large and complex pigmented pecten. The position of this organ (*cf.* Krause, 1922, Fig. 88) is such that it, or rather the presence of its serrated shadow on the retina, may well be expected to have an effect upon the sensory influence of moving images, as suggested by Menner (1938), although in a recent review of this matter Walls (1942, pp. 367, 520, 648) concludes it to be unlikely that the pecten casts a shadow outside its own base or elsewhere "where it would do any good." No specific signs of an influence of the pecten could be detected in our measurements with $t_L = 0.50$. The symmetry of the $F - \log I$ contour was not disturbed, and subsequent series of observations at $t_L = 0.10$ with lights from different spectral regions have confirmed this (Crozier and Wolf, 1941-42 *d*). The very low intensity level at which the $F - \log I_m$ curve is located, by comparison with that for other vertebrates, is sufficiently accounted for by the bird's higher temperature (Crozier and Wolf, 1940-41 *b*), and requires no assumption that the presence of the pecten is responsible.

We were led to believe that study of the modification of the $F - \log I$ curve by systematically changing the light-time cycle fraction (t_L), employing the methods already used with various animals (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *d, e*; Crozier and Wolf, 1940, 1939-40 *b*, 1940-41 *d*), might reveal a distinctly unusual situation. The comparative morphology of the pecten, and the complexity of its shadow on the background of the eye in diverse birds of different habits, strongly suggests (Menner, 1938) that the effect of the pecten might be to increase the sensory action of small moving images. Con-

sequently, the particular effect (if present) should be especially apparent, with the striped cylinder technic, when using narrower dark stripes and broader light spaces ($t_L > 0.50$). The proof that this is the case is contained in the present paper. The systematic changes in the $F - \log I$ contour as a function of t_L are with the zebra finch of a kind not found in other animals lacking a well developed pecten.

The avian pecten is a membranous organ, highly vascular, rather densely pigmented, attached to the retinal surface of the eye along the entrance of the optic nerve. It projects for a considerable distance into the vitreous humor. Its surface is elaborately plicated. In our birds the ophthalmoscopic appearance of the eye agrees rather well with Wood's account for members of the related Fringillidae (Wood, 1917, Plate LIV); the eyeground is bright blue-green-gray, with bright dots. A great variety of guesses has accumulated concerning the functional significance of the pecten,—that it is concerned with the nutrition of the vitreous, or with excretion (*cf.* Johnson, 1901, 1927); that it is an erectile organ, and may be involved in the movement of liquid during lens movements and the accommodation of the eye; that it is a visual shield, especially for that part of the field in front of and above the bird's head, and that it may therefore serve to suppress *binocular* vision; or that it protects the retina from too great illumination; or that it is a sense organ. The notion must be guarded against that the pecten, or any organ, should have a function. The degree of its development in different birds is apparently coordinated with the dominance of photopic vision and with the use of monocular rather than binocular perception. During ophthalmoscopic observation the impression is very easily gained that the pecten moves about within the vitreous. The incorrectness of this notion was demonstrated by Beauregard and by Paul Bert, for pigeon and duck, in 1876. We can confirm their conclusion that the apparent larger movements of the pecten are actually due to the movements of the eyeball; the vibratory pulsations of the pecten which can be detected are mechanically transmitted to the eyeball by the contraction of the muscle which operates the nictitating membrane. There remain quite minor pulsations of smaller amplitude which may well be due to fluctuations in the circulatory pressure. We find in our quantitative evidence on the reaction to flicker no indication that the functioning of the pecten as a "light shield" introduces any modification of the fundamental nature of the flicker response contour. The effects actually found are of quite another sort.

The demonstration that the highly exceptional properties of the zebra-finch $F - \log I$ curve to be described in relation to t_L are really due to the presence of the pecten shadow cannot be made by removing the pecten. Nor can a veritable pecten be put into the eyes of other forms. But the same effect can be produced by projecting an appropriate shadow on the human retina. It is shown that in this way the specifically peculiar properties of the bird's flicker

response contours can be essentially reproduced. The experiment provides a proof of the visual significance of the avian pecten. It also opens the way to the experimental analysis of the problem of visual integration through the use of subdivided patterns, of which we shall have something to say in later papers.

The consequences of breaking up a visual area (in man) into several parts can give a proof of the separability of the two general factors we have conceived to be operating in the production of a given level of visual excitation, namely (1) the number of neural units concerned and (2) the mean number of elements of sensory effect produced in each unit. Some practical consequences will be briefly referred to. Since the pecten effect is one apparently directly involving only the "cone" response contour, it becomes possible to separate experimentally the human "cone" and "rod" contributions, with a result confirming in important respects the conclusion from previous analytical interpretations.

II

With an insect (*Anax*, nymph), sunfish (*Enneacanthus*), turtle (*Pseudemys*), and man, reduction of the light-time fraction in a flash cycle of given form increases $F_{max.}$ of the $F - \log I$ curve and decreases the abscissa of inflection, each in rectilinear proportion to t_L . The third parameter, $\sigma'_{\log I}$, the S.D. of $dF/d \log I$ with $F_{max.}$ put = 100, is not modified at all. The statistical basis for the generality of this rule has been indicated in earlier discussions. The S.D. parameter is demonstrably (in man) a function of the number of available neural units, while $F_{max.}$ measures the total number of elements of sensory effect producible under the conditions. Reduction of the t_L fraction means that, in virtue of the correlated increase of the percentage dark-time, each flash has a greater chance of finding less refractory units to work upon; hence a given level of effect ($\propto F$) is achieved with a lower flash intensity, although the total number of available and participating units is not changed.

The observations with the zebra finch were made in the way described in our account (Crozier and Wolf, 1960-41 *b*) of the flicker response contour with white light at $t_L = 0.50$, by the use of the initiation of head nystagmus as an end-point. Series of striped cylinders providing $t_L = 0.10, 0.25, 0.50, 0.75$, and 0.90 were used in the present tests. The method and general procedure are discussed in the paper by Crozier and Wolf, 1939-40 *b*. Four selected individual male birds were used throughout. No consistent differences were noticed in the excitabilities of these four. (Two females were also used at $t_L = 0.50$ and 0.90 ; cf. Table II.) At each flash frequency F , three observations were made of the critical flash intensity I with each bird, averaged, and the means and P.E.'s of these averages appear in Table I.

The relations of $\log I_m$ to flash frequency F are shown in Fig. 1. The set of curves does not have the character found for *Anax*, sunfish, turtle, and man. From $t_L = 0.10$ to 0.50 , $F_{max.}$ declines and τ' increases pretty much in the regu-

lar way; but with $t_L = 0.75$, and more extremely with 0.90, the asymptotic maximum is *increased*, τ' is less, and the slope is markedly greater, so that the two latter curves cut across the other three.

Despite the changing shape of the $F - \log I$ contour with alteration of t_L , the curves are still well described by a normal probability integral (Figs. 1 and

TABLE I

Critical intensities (white), as $\log I_m (ml.)$, for response to flicker by the zebra finch (*Taeniopygia castenotis*), with different proportions of light-time (t_L) to cycle time ($1/F$), as a function of flash frequency F . Each entry is the mean of twelve measurements, three on each of four male birds. Under $t_L = 0.50$ entries are also given in Fig. 1 from a previous experiment (Crozier and Wolf, 1940-41b) with other individuals. Each entry is accompanied by its $\log P.E.$.

F per sec.	$t_L = 0.10$		0.25		0.50		0.75		0.90	
	$\log I_m$	$\log P.E.$	$\log I_m$	$\log P.E.$	$\log I_m$	$\log P.E.$	$\log I_m$	$\log P.E.$	$\log I_m$	$\log P.E.$
2			6.1953	8.7376			5.1153	7.1867	6.4338	7.5304
3	6.2975	8.6992								
5	6.6734	8.9905	5.0253	8.9059			5.6302	7.5273	5.8069	6.2471
7	5.0141	7.3219							5.9805	6.0950
10	5.4033	6.2016	5.6988	7.8468			4.1741	6.7278	4.1738	6.9873
	5.3747	7.9649							4.2445	6.9189
15	5.9076	6.0722	4.2106	6.5458			4.4807	6.7766	4.4140	6.7096
20	4.3822	5.0358	4.6375	5.0933	3.0849	5.2253	4.7613	6.7088	4.6571	6.7200
	4.4104	5.1084	4.6756	5.0425	3.1550	5.5870	4.7426	6.9497	4.6489	5.2555
25	4.8285	5.1416	3.0663	5.3065			3.0565	5.1656	4.8633	5.0416
30	3.1326	5.7585	3.3876	5.9905	3.8460	5.3784	3.3838	5.6279	3.0820	5.6378
			3.4621	4.1034			3.3916	4.0907		
35	3.5031	5.9033	3.7962	4.1888			3.7017	4.0958	3.2470	5.4702
40	3.8432	5.7239	2.0803	4.6279	2.6536	4.9350	2.0086	5.5334	3.4244	5.7415
			2.1517	4.3368			2.1605	4.3830		
45	2.3210	4.6639	2.6928	4.9352			2.2903	4.6906	3.6402	5.9905
50	1.0966	3.3786	1.4574	3.1583	1.9553	2.0250	2.6373	4.8407	3.9255	4.3209
			1.4468	3.6592			2.6280	3.0695		
53	1.8084	2.1976	0.1827	2.7318			2.9929	3.4327		
55	0.9613	1.1100	1.3593	1.7357	2.0390	1.7752	1.9658	2.0776	2.6472	4.7748
			1.3705	2.9401			1.9763	3.9006		
56.5									1.1216	2.0065

2). The slight departures are nevertheless systematic. Near the inflection point the departures tend to be in excess, and half way above it in defect. This is clearer below $t_L = 0.75$. Although any one of these departures is only barely significant statistically, their reality is attested by their further occurrence in our data with colored lights (Crozier and Wolf, 1941-42 d). Several factors are possibly concerned in producing them. For the control of light intensity in the apparatus it was necessary to use filters; these are very nearly

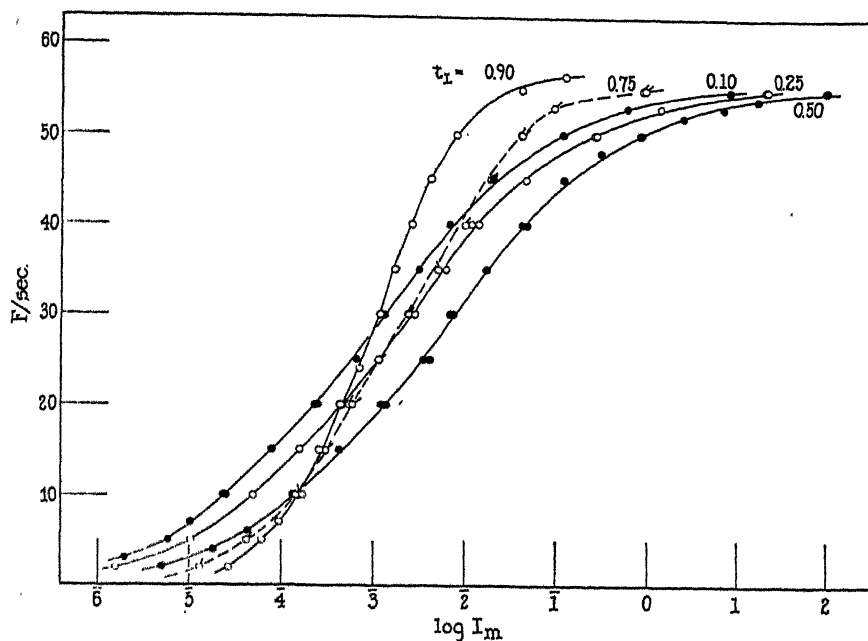


FIG. 1. Flicker response contours for the zebra finch (*Taeniopygia castanotis* [Gould]) with different light-time fractions in the flash cycle, 0.10 to 0.90. White light. Data in Table I. The curves drawn are probability integrals (Fig. 2).

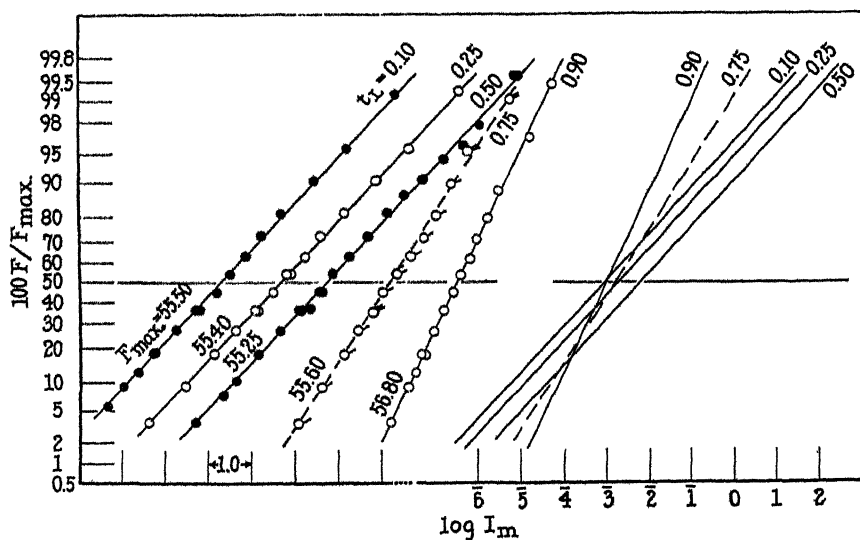


FIG. 2. The data of Fig. 1 on a probability grid. At the left the different curves are separated arbitrarily for clearness. On the right the lines drawn are shown in their natural relative positions.

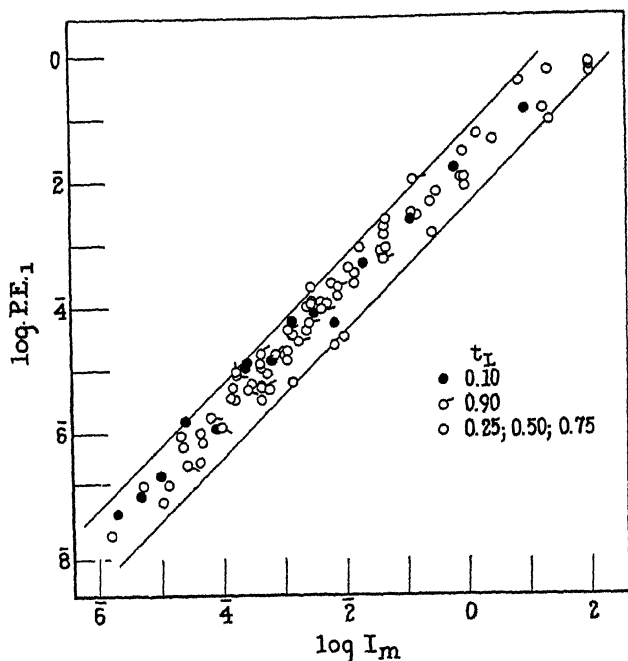


FIG. 3. The proportionality between I_m and $P.E.1$ in the data of Table I is direct (that is, on the double log grid the points form a band of unit slope). The proportionality constant does not significantly depend on t_L .

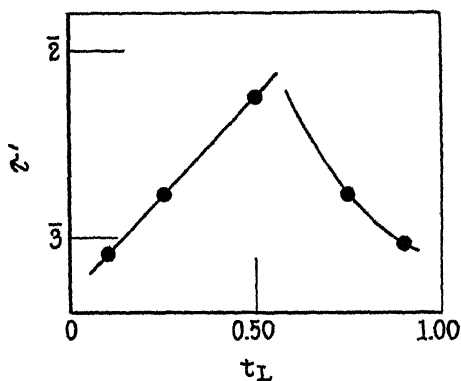


FIG. 4. The abscissa of inflection (r') of the curves in Fig. 1 is directly proportional to the percentage light-time from $t_L = 0.10$ to 0.50; for higher values of t_L it becomes smaller.

“neutral” for the normal human eye, as shown by spectrophotometric measurements, but are not necessarily so for the bird. Again, in covering the range of

flash frequencies use is made of rotated cylinders with 5, 10, 20, or 40 stripes, in different parts of the range. There is no absolute correlation of the slight systematic departures with either of these influences, but it is possible that in view of the action of the pecten they may play a part. There seems no basis for suspecting that the iris is involved in the departures, although they seem lessened at $t_L = 0.90$.

The variation of I_1 as a function of I_m shows no real correlation with t_L (Fig. 3).

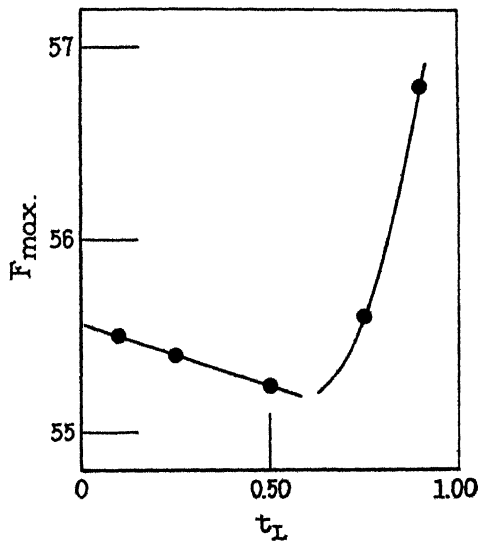


FIG. 5. F_{max} . for the curves in Fig. 1 declines with increase of t_L up through $t_L = 0.50$, thereafter increases.

The change of τ' with t_L is shown in Fig. 4, and of F_{max} . in Fig. 5. The changes are rectilinear in t_L up to 0.50, then alter direction abruptly. This is correlated with the constancy of $\sigma'_{\log I}$ for $t_L = 0.10, 0.25$, and 0.50, and its abrupt decrease beyond that point (Fig. 2).

III

The interpretation given for the t_L data in cases thus far known was applied to situations in which the retina is comparatively free from fixed shadows. The eyes of the gecko and of *Phrynosoma* have minute simple pectens, it is true; but that of the zebra finch is very much more elaborately developed. In each of the instances mentioned the simplex flicker response contour is quite symmetrical (Crozier, Wolf, and Zerrahn-Wolf, 1938-39 *a*; Crozier and Wolf, 1938-39 *b*, 1939-40 *b*). This is consistent with the present findings, and has an important bearing on the interpretation of visual data in general (*cf.*

section V). But in the presence of a fairly elaborate pecten shadow it is clear that the parameters of the flicker response function (moving stripe method) suffer a kind of dependence on t_L which is not otherwise found. The experimental proof that the pecten shadow does involve this sort of effect is given in section IV.

By testing separately and in combination the influence of image area, location, exposure time, temperature, light-time fraction, and wave-length composition it has been shown that three independently modifiable parameters are required for the description of the visual excitability function (*cf.* Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *a*; Crozier, 1939; 1940 *a*, *b*; etc.). For the "cone" curves it is found that increasing the number of *cone* units available for excitation causes a *decrease* in $\sigma'_{\log r}$ (*cf.* Crozier, 1940 *a*; Crozier and Wolf, 1941-42 *b*). This is the effect found with the zebra finch at $t_L = 0.75$ and 0.90. On the other hand, F_{max} is also increased by augmenting the mean contribution from each unit, but without change in $\sigma'_{\log r}$. It may thus tentatively be deduced that with narrow dark bands and wider light bands, as in our tests with longer light-time intervals, the production of the end-point effect for the bird is due to the activation of a larger number of neural units, regardless of whether the excitation of these units is then more effective in the sense that larger numbers of elements of effect are obtained from them. This means that F_{max} must tend to pass through a minimum as t_L is increased (Fig. 5). The pecten shadow produces a serrated light-dark line along which contrast is enhanced. The interruption of light by superimposed dark-light transitions moving across this pecten shadow must be expected to produce an enhancement of the sensory effect if the dark intervals are brief by comparison with the alternating light intervals. Consequently the end-point effects must for short dark-times be expected to increase, especially along the illuminated edge of the pecten shadow. This corresponds exactly to the subjective effect when the human observer is provided with a "pecten shadow" on the retina (see section IV). It is thus easily explained why the unusual effect seen in Fig. 1 occurs only with short dark-times, and is greater with $t_L = 0.90$ than with 0.75. When such comparatively small dark-times are used the retinal effective area is enlarged, an increased contribution to the end-point effect then arising along the margin of the pecten shadow because the units there located are involved in a contrast situation which enhances their neural efficiency.

The phrasing of this interpretation implies that the neural effect determining the end-point response is taken to be located central to the retina. In this connection we have to note that for all the light-time fractions used the curve is smoothly symmetrical. It is pointed out subsequently (sections IV and V) that the same thing is found when a number of flickered patches on the human retina are separated by darkness. The synthesis implied cannot very well be understood unless a central nervous locus be accepted for it.

The changing form of the $F - \log I$ curve as a function of t_L cannot be attributed to a rôle of the bird's iris. Several kinds of evidence support this assertion. With no pupil correction possible or required, the corresponding data with *Anax*, *Emneacanthus*, and *Pseudemys* show the constancy of $\sigma'_{\log I}$ when t_L is altered, just as with man when an effective artificial pupil is used. With elimination of the pupil variable and the introduction of a "pecten shadow," the human curves (section IV) show as a set the properties of those seen in Fig. 1. It is also consistent with this that, in the case of the zebra finch, $\sigma'_{\log I}$ with t_L constant is not noticeably a function of spectral region (Crozier and Wolf, 1941-42 d).

In passing it may be remarked that if one were to attempt the interpretation of the form of the flicker response curve from the standpoint of the theory of the retinal photostationary state (Hecht, 1937), and if one had in hand only one of the curves of Fig. 1, the system of reaction-orders one would assume would necessarily be quite different say for $t_L = 0.10$ and 0.90 ,—neglecting the fact that the equations do not really describe the full range of the data in any case (cf. Crozier, Wolf, and Zerrahn-Wolf, 1938-39 a, b; Crozier and Wolf, 1939-40 a). This is equally true for the curves in the corresponding experiment with man (section IV). The direction of the shift of the curve with increase of t_L , of course, negatives the whole conception in any case (Crozier, Wolf and Zerrahn-Wolf, 1937-38 d, e, etc.).

From these findings it is apparent that a definite functional significance can be assigned for the pecten: it increases the sensory action of small moving shadows. This is entirely in agreement with deductions made on the basis of the nature of the pecten shadow in birds of different visual habits, and from some not altogether conclusive experiments with man (Menner, 1938). In sections IV and V it will be shown that no *direct* effect of a "pecten shadow" can be demonstrated for the "rod" $F - \log I$ curve in man. This might be correlated with the small size of the pecten in nocturnal birds (cf. Wood, 1917; Franz, 1934; Menner, 1938). It can be tested in a more significant way by examining the responses of a bird exhibiting visual duplexity, as we do in the following paper. Another kind of test is also possible, because if the theory is sound the "pecten" effect should be reduced by using a flash cycle of different wave form in which the light-dark transitions are less abrupt.

The pecten is probably not stationary; its slight movements in addition to those of the head as a whole, and of the eyes, may well play a part in causing small images to sweep across the retina. The same general enhancement of acuity for smaller images should be produced by movement of the pecten shadow even if the image and the eye as a whole are stationary. This we have tested for the human eye, with interesting indications already at hand. It is not impossible that the lobulated iris of some forms (e.g., camel, goat) may function in a similar way. One thinks also of the experience of those who have

had to do with long-haired dogs; there is a general impression that the hair in front of the eyes is actually a visual aid. The present interpretation of the visual rôle of the pecten shows that this is not implausible. (A suggestion of a different sort has been made by Swindle (1917) for the function of the "eye appendage" hairs of the cat and other animals, namely that they aid ocular fixation.) The matter can be tested with dogs. Finally, the thought occurs that the barred or patched patterns of the males of certain types (as the zebra finch) exhibiting pronounced sex dimorphism could conceivably be connected with a sex differential development of the pecten or of its effect. We mention this because the data of Table I were obtained exclusively with *male Taeniopygia* (some 17 individuals). However, careful tests with two females at $t_L = 0.50$ and 0.90 (Table II) show no trace of significant divergence from the measurements with males (Crozier and Wolf, 1940-41 *b*, and Table I of the present paper).

TABLE II

Critical flash intensities (white) for $t_L = 0.50$ and 0.90, with 2 *female* zebra finch; each I_m is the average of six measurements. Cf. Table I, and text.

F per sec.	$t_L = 0.50$	0.90
	$\log I_m$	$\log I_m$
10	4.12	4.18
20	3.12	4.68
30	3.86	3.08
40	2.65	3.42
50	1.96	3.94

IV

With a test area of sufficient size, the human $F - \log I$ contour exhibits the fundamentally duplex character of the typical vertebrate visual mechanism. The curve is a complex of two partially overlapping S-shaped components (cf. Hecht and Verrijp, 1933; Hecht, Schlaer, and Smith, 1935; Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b*; Crozier and Wolf, 1941-42 *b*). The essential properties, and the quantitative form, of the "cone" segment of the duplex curve are the same with different methods of producing flicker (Crozier and Wolf, 1940-41 *c*; Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *b*). The low intensity segment of the duplex curve is ordinarily the additive resultant of "rod" and "cone" contributions. By means of observations in two different regions of the retina, involving the use of t_L and of wave-length composition as variables (Crozier and Wolf, 1940-41 *d*, 1941-42 *a*, *b*), it has been demonstrated that the separation of the essential "rod" contribution to the $F - \log I$ contour can be successfully effected analytically by the procedure already used for various other vertebrates (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 *a*, *e*, 1939-40 *c*; Crozier and Wolf, 1938-39 *a*, 1939-40 *a*), including forms

in which the overlapping of "rod" and "cone" effects is not so complete. The $F - \log I$ curve is the same, and its changes with t_L are the same, when flicker is produced (1) by sectoring a light beam at a focus (Crozier and Wolf, 1940-41 c) and (2) by the use of the striped cylinder technic; in the latter case the eye of the observer is optically placed within the striped cylinder by the use of telescope and prisms (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 b).

A distinction must be carefully made, however, between certain other properties of the two stimulus situations just mentioned. Thus when flicker with a subdivided field is produced by method (1), the change of τ' as a function of t_L is of the type about to be described and already seen in the tests with *Taenopygia*; but $\sigma'_{\log I}$ does not then change at all (Crozier and Wolf, 1943-44 b), in sharp contrast to the case with procedure (2) when a "pecten shadow" is present. It scarcely requires emphasis that tests of this kind demonstrate qualitatively the existence of essentially three independently modifiable parameters in the equation of the flicker contour. (It will be of considerable interest to learn whether in these two cases there appear differences in the be-

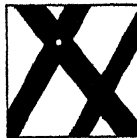


FIG. 6. Diagram of the flickered field produced by an opaque grid (see text). The field subtended a visual angle of 14.3° on a side at the retina. The small circular dot served as foveal fixation point.

havior of the contours with respect to the influence of temperature, for example, or oxygen pressure.) In any case, we have evidence supporting the conception of the importance of "sliding contact" between moving images and the margins of the pecten shadow.

We desired to create a condition for the human observer which would mimic in certain essentials the normal state of the eye in a bird with well developed pecten shadow. To do this we provided in the field of view of our "periscopic" telescope (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 b, Fig. 1) a cross-barred diaphragm of copper foil. Its appearance is given by the scaled drawing in Fig. 6. The slanting angle of the bars was arranged to give inclined edges across which the vertical images of the revolving cylinder stripes would pass. At the point indicated in Fig. 6 a minute hole provided a fixation point. Thus the image field fell chiefly on the temporal side of the fovea and below the horizontal meridian.

The square field as a whole subtended an angle of 14.3° at the retina. The illuminated parts of the field aggregated *ca.* 140.2 square degrees, or 59.8 per cent of the total square field. It is of interest that, as shown subsequently, $\sigma'_{\log I}$ is a little greater than for the full 14.3° field centrally fixated, in correla-

tion with its smaller size; this cannot be fully interpreted, however, until more is known of the process whereby the synthesis of critical effects from distinct, simultaneously flickered patches is effected.

Data were obtained with two observers, monocularly (left eye). As in previous measurements with this particular apparatus, no pupil correction is

TABLE III

F vs. $\log I_m$ for W.J.C., left eye, white light, 14.3° square with "grating"—see text; ten observations at each point; I_m and P.E.₁ in $ml.$, for five values of the light-time fraction.

F per sec.	$t_L = 0.10$		0.25		0.50		0.75		0.90	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2			7.9309	8.8629	6.4338	7.0605	6.8953	7.5572	5.5363	7.5616
4			6.2268	8.5894	6.7323	8.9593	5.1808	7.5509	5.8444	6.7486
6	7.8525	8.3508	6.4726	8.6659	6.9413	7.1110	5.3668	7.7374	4.0777	6.1052
8	6.0867	8.5157	6.7091	7.1067	5.1818	7.9226	5.6414	7.6709	4.3164	6.8978
10	6.3558	8.7409	6.9336	7.1424	5.4475	7.9375	5.9028	6.2187	4.6424	6.9818
					5.4393	6.1052			4.6518	5.1708
12	6.5711	8.8426	5.3075	7.8864	5.6679	6.0060	4.1425	6.6302	4.8660	5.0901
14	6.8581	8.8036	5.5616	7.8319	5.9401	6.1780	4.4173	6.7309	3.3326	5.9046
16	5.3659	7.8353	5.9618	6.2040	4.4548	6.8664	4.9048	5.1464	3.7669	4.2229
18	4.7619	5.0955	3.0128	5.4631	3.8446	4.5138	2.3272	4.6506	1.1089	3.3780
20	3.4539	5.9894	3.9403	4.2811	2.5816	4.9511	1.0298	3.5762	1.6452	2.5293
	3.4521	5.8048	2.2235	4.6799	2.5599	3.2492	1.0414	3.0770	1.6702	3.9554
25	2.0445	5.9230	2.6610	3.0629	1.1294	2.9875	1.4824	3.7352	1.9652	2.7669
30	2.3576	4.8386	1.0023	3.3766	1.4765	2.5864	1.7544	3.9858	0.0874	2.5476
35	2.8333	4.8949	1.4326	3.4749	1.9019	2.5887	0.1113	2.1052	0.3467	1.0708
40	1.3640	3.8420	1.9771	2.1441	0.4864	1.1311	0.4257	2.6839	0.6798	1.3319
43									0.8786	1.1610
45	1.8841	2.1325	0.4923	2.7881	0.9869	1.5506	0.8091	2.8347	1.2707	1.6807
					0.9564	1.5437			1.2721	1.8072
					1.2674	1.8983			1.8167	0.0312
47										
48	0.4381	1.0358	1.0088	1.0327			1.2206	1.2659		
50	1.2025	1.3154					1.9060	1.5973		
51.0			1.8269	1.9495						
51.5	1.8926	1.6827								

required; the reason for this (although not stated in our earlier paper, as it should have been; Crozier and Wolf, Zerrahn-Wolf, 1937-38 *b*) is that the diameter of the light beam at the eyepoint is about 1.8 mm. The measurements are given in Tables III and IV, as $\log I_m$ and $\log P.E._1$ (millilamberts). The flicker response contours are plotted in Figs. 7 and 8.

It is apparent that the presence of the "pecten" grid produces a set of human $F - \log I$ contours with t_L as variant which in a qualitative sense imitates the peculiarities of the set obtained with zebra finch (Fig. 1). In the usual situation, with no "pecten shadow," the curves obtained for the several values of

t_L form a group in which the "cone" segments have the same shape constant $\sigma'_{\log t}$ (Crozier and Wolf, 1940-41 *d*). This is obviously not the case in Figs. 7 and 8. Quantitatively, the distortion is not the same for the two observers. For W.J.C., with larger $\sigma'_{\log t}$ and lower values of $F_{max.}$, although lower values of τ' , and consequently a proportionately smaller number of elements of neural action produced, the pecten effect is relatively less pronounced. This might

TABLE IV

F vs. $\log I_m$ for E.W., left eye, white light, 14.3° square with "grating"—see text; ten observations at each point; I_m and P.E.₁ in *ml.*, for five values of the light-time fraction.

F per sec.	$t_L = 0.10$		0.25		0.50		0.75		0.90	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2			6.0265	8.8894	6.4301	7.1757	6.8014	7.3832	5.2862	7.8102
4			6.3195	7.0291	6.7077	8.9936	6.0839	7.5476	5.5442	6.1410
6	6.1778	7.0815	6.6048	7.4095	6.9872	7.5415	6.3574	6.1385	5.8132	6.3905
8	6.3960	7.0387	6.8233	7.3533	5.1986	6.1519	5.5992	6.1637	4.0927	6.4093
10	6.7011	7.2553	5.1284	7.4989	5.5069	6.0117	5.8925	6.2898	4.3600	5.2038
12	5.0569	7.5498	5.6001	6.1337	5.8873	6.4310	4.2876	6.9898	4.7212	5.2915
14	5.7134	6.0789	4.1474	6.4001	4.5313	5.1956	4.9047	5.4672	3.3249	4.1776
16	3.2477	4.1876	3.6721	4.3535	2.1014	4.6497	2.4853	3.0831	2.8972	3.3939
18	3.5764	4.1836	2.0306	4.5574	2.4980	3.2433	2.7782	3.1220	1.2350	3.5992
20	3.8032	4.3579	2.2822	4.5311	2.6474	3.3246	1.0004	3.8262	1.4636	2.1956
	3.8023	4.4874	2.3079	3.0908	2.6507	3.5571	1.0021	3.4138	1.4733	3.8370
25	2.2594	3.0412	2.6483	3.4792	1.0056	3.0540	1.3274	3.6879	1.7693	2.2844
30	2.6760	3.0163	1.0924	3.9221	1.4785	2.0952	1.6551	2.0053	1.9726	2.4940
35	1.1467	3.4299	1.6076	2.2192	1.9736	2.5785	1.9817	2.3147	0.1801	2.6583
40	1.6969	2.1776	0.0917	2.6229	0.5173	1.0106	0.2887	1.0724	0.4857	1.1175
45	0.1626	2.5001	0.5696	1.2676	0.9635	1.3253	0.6083	1.2585	0.6739	1.4464
48					1.3062	1.7111				
50	0.7146	1.2435	1.1264	1.5606			1.0726	1.5476	1.1912	1.7568
52	0.9888	1.2066	1.3927	1.8642	1.8762	1.9331			1.9083	1.9193
53							1.8993	1.7938		
55			1.9548	0.0288						
56	1.8569	1.8778								

well be taken as a difference entirely in keeping with the analysis proposed in section III. It will be pointed out in the detailed consideration of the data of Figs. 7 and 8 that there is no reason to suspect a specific influence of the "pecten" shadow on the curves of "rod" performance, which perhaps accords with the general conception of the association of the development of the avian pecten with the accentuation of cone efficiency. The slight changes in the "rod" curves can be shown (section V) to be due secondarily to the interaction of "rod" and "cone" effects. The same is true in our data on the sparrow (Crozier and Wolf, 1943-44 *b*). On the basis of the differences between Figs. 7 and 8 there would seem to be no doubt that in other individuals more or less

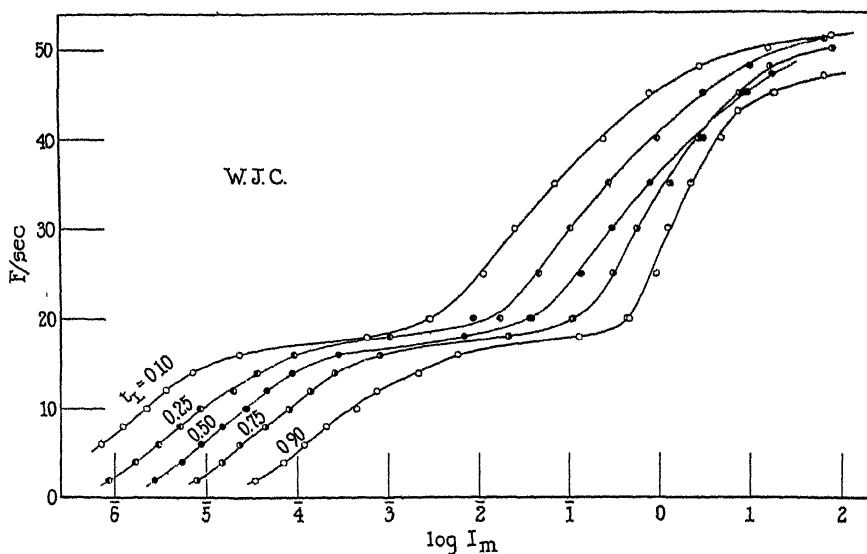


FIG. 7. The measurements of Table III (W. J. C., left eye) giving flicker recognition contours (moving stripe method) with different light-time fractions (t_L) using the divided field diagrammed in Fig. 6.

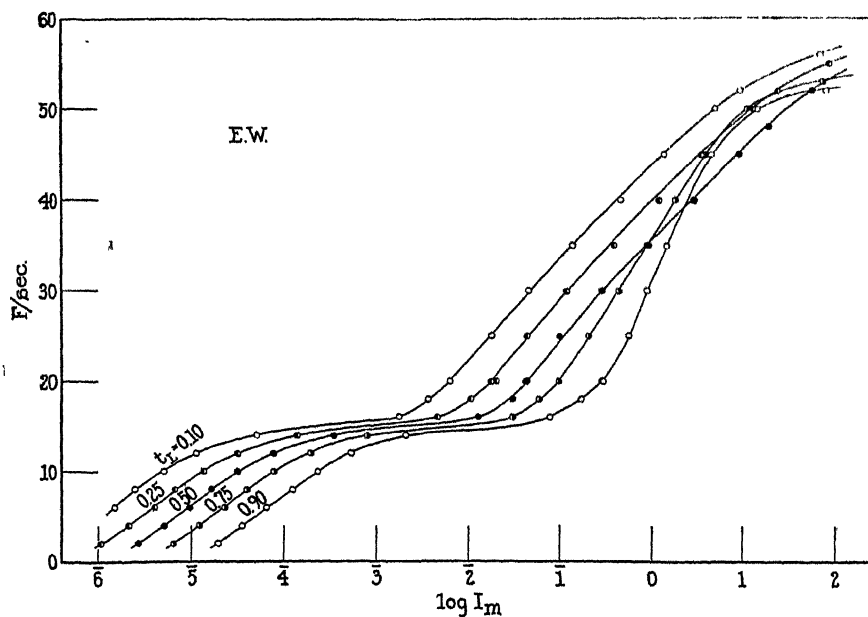


FIG. 8. The measurements of Table III (E. W., left eye) giving flicker recognition contours (moving stripe method) with different light-time fractions (t_L) using the divided field diagrammed in Fig. 6.

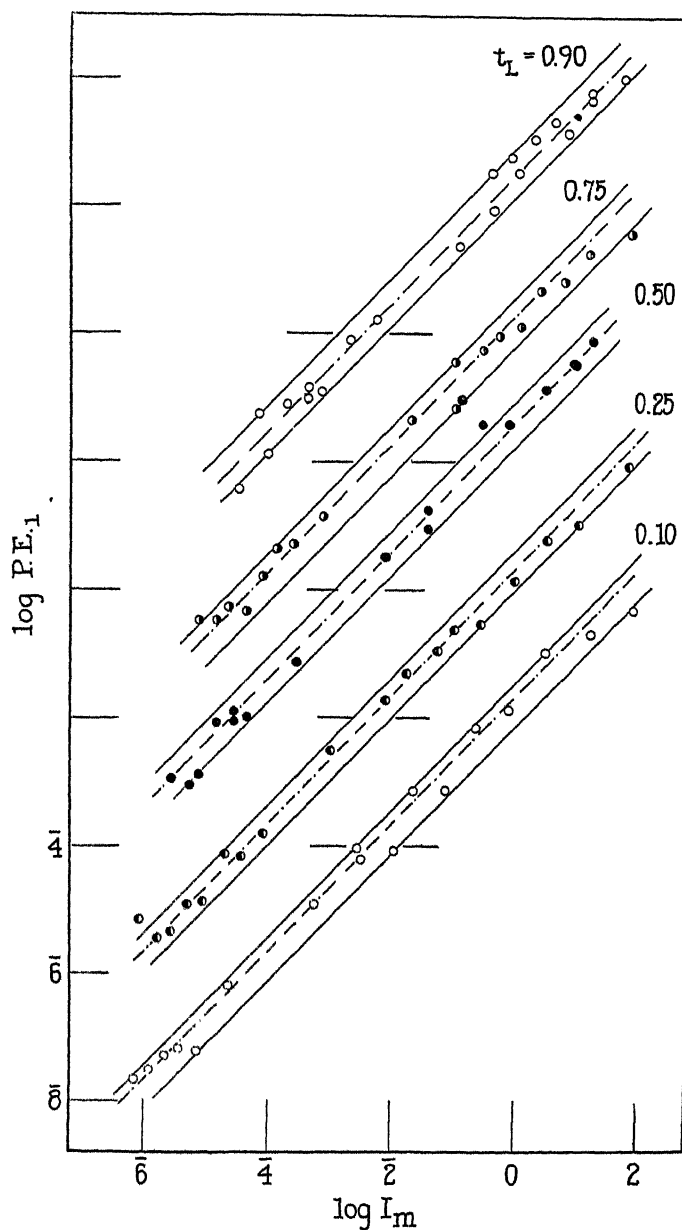


FIG. 9. $\log I_m$ and $\log P.E._1$ are rectilinearly related with a slope of 1, in the data of Fig. 7.

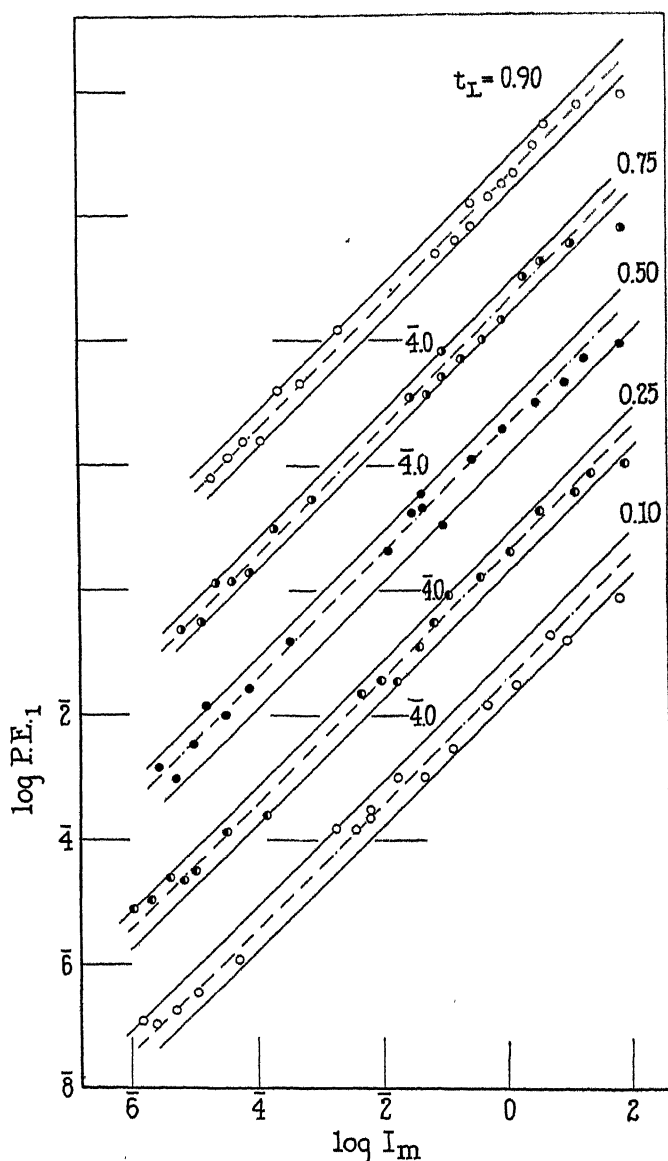


FIG. 10. $\log I_m$ and $\log P.E._1$ are rectilinearly related with a slope of 1, in the data of Fig. 8.

extreme manifestations of the "pecten effect" could be found. There would also seem every reason to believe that with other kinds of "pecten" shadow more pronounced distortions could be produced. Still it is of interest that,

as with the bird, the curves for t_L 0.10, 0.25, and 0.50 have the same slope constants and that their F_{max} and τ' change with t_L in the normal way; only with t_L extended to 0.75 and 0.90 is the distortion obvious. In all these respects the human "cone" curves, when the "pecten" shadow is present, behave like those found with the zebra finch. The general parallelisms with the bird measurements strengthen the basis for speaking of the response contours obtained with lower animals as *flicker* contours.

In one other respect, also, the data have similar properties. In Figs. 9 and 10 the variation of flash intensity critical for flicker is considered as a function of the mean critical intensity at various levels of F . For each observer the mean value of $P.E._1/I_m$ is independent of t_L , but the breadth of the scatter band of $P.E.$'s tends to be greater at $t_L = 0.50$. The mean values of $P.E._1/I_m$ (and of its scatter) run consistently a little higher for E.W. than for W.J.C. This has been noted in certain other experiments as well. The average precision of the measurements as plotted may be given as $\sigma_m = 1.3$ per cent of I_m . This value is lower by half than that obtained in our earlier and less homogeneous series with this instrument (Crozier, Wolf, and Zerrahn-Wolf, 1937-38 b). In Figs. 9 and 10 no attempt is made to take account of the slight tendency of the upper points to "drop off"; the highest point tends to be low because of the manipulative procedure involved at the highest intensities with this apparatus.

The "cone" curves drawn in Figs. 7 and 8 are the upper portions of probability integrals, shown rectified in Figs. 11 and 12. In these latter plots the curves have been arbitrarily separated for clearness; their correct relations to one another are given in Figs. 13 *a* and *b*. The maximum ordinates to which these curves have been computed are shown in Fig. 14 as a function of t_L . The relations shown, and for τ' as well (Fig. 15), are not the same as found in the bird data (Figs. 4 and 5). It is apparent that instead of declining rectilinearly with increase of t_L , F_{max} here tends to rise slightly, then sinks rapidly. This is taken to mean that, in the human case, even at light-time fractions less than 0.50, the presence of the "pecten" shadow used tends to increase the production of elements of neural effect; since $\sigma'_{\log I}$ does not change appreciably (only for E.W. is it slightly diminished at $t_L = 0.50$), this is held to indicate that with increase of t_L up to 0.50 and possibly beyond there occurs a more effective "summation" of excitations from the several illuminated patches (Fig. 6). With further increase of t_L beyond 0.50 the significant location of the end-point flicker, subjectively, is predominantly along the edges of the crossed bars, and the number of active cone units is correspondingly enhanced, with consequent reduction of $\sigma'_{\log I}$. The same considerations account for the behavior of τ' . From this standpoint the properties of the sets of curves in Figs. 7 and 8 are complicated by a phenomenon not encountered in the bird data, namely the "summation" effect. This is not surprising, since the bird's pecten shadow

does not isolate patches of illumination, but merely provides a serrated shadow outline.

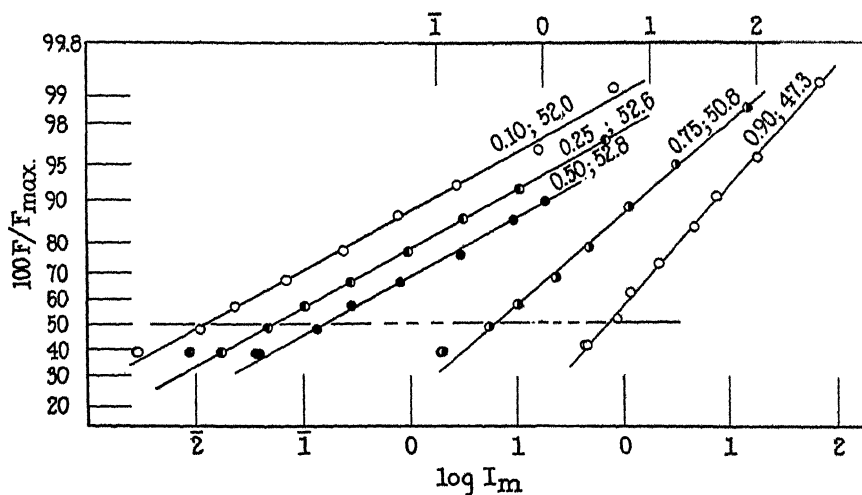


FIG. 11. The upper segments of the curves in Fig. 7 shown on a probability grid, the several curves separated laterally for clearness.

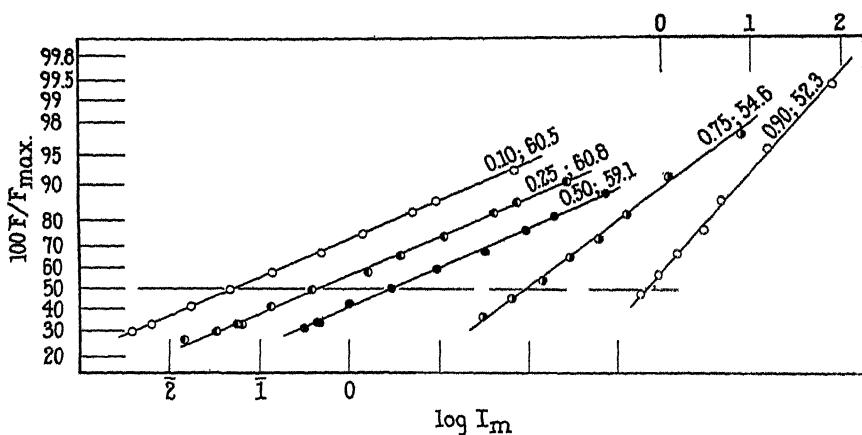


FIG. 12. The upper segments of the curves in Fig. 8 shown on a probability grid, separated for clearness.

We must note here that the present evidence for invoking summation on the basis of flicker end-points is not at all of the kind urged by Granit (1930) as arguing for (extrafoveal) retinal summation. In Granit's observations it

was found that the fusion frequency for four small patches in the periphery was a little higher than that for one patch, at the same flash intensity. This result is almost inevitable, even if there were no question of the involvement of scattering of light in the eye, because the total area covered by the four spots is

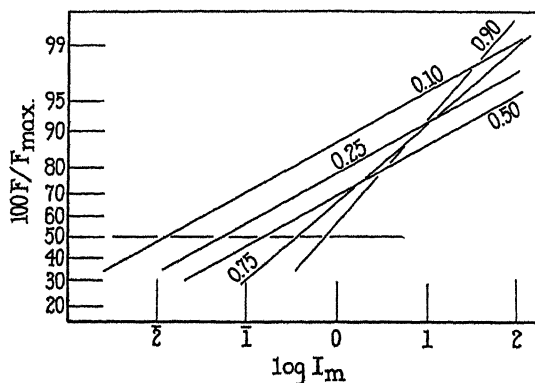


FIG. 13 a

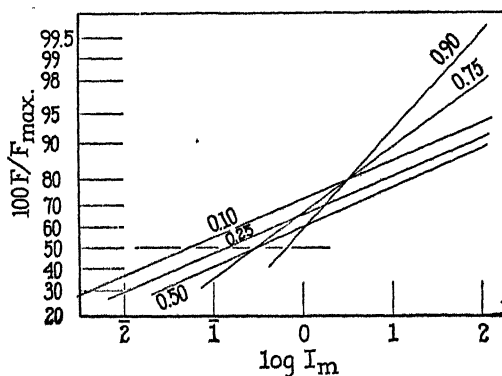


FIG. 13 b

FIG. 13 a and b. The lines of the graphs in Figs. 11 and 12 shown in their natural relationships.

greater than that covered by one of them. It is well known (Granit and Harper, 1930, etc.) that increasing the size of the test image produces just this effect. A proper examination of the question of "retinal integration," which Granit's experiment does not give, requires a more elaborate investigation of the flicker response contour when the area effect *per se* can be ruled out, and with due attention to the possibility of distinguishing between monocular and

binocular integration effects (*cf.* Crozier and Wolf, 1940-41 *c*). This we propose to undertake elsewhere.

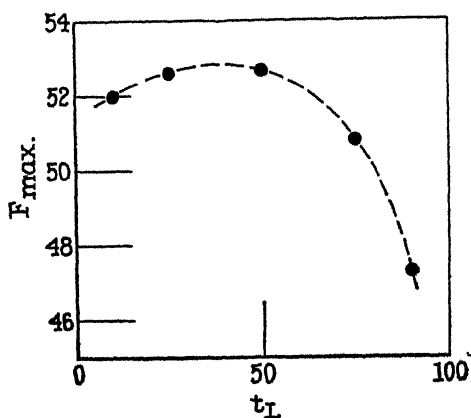
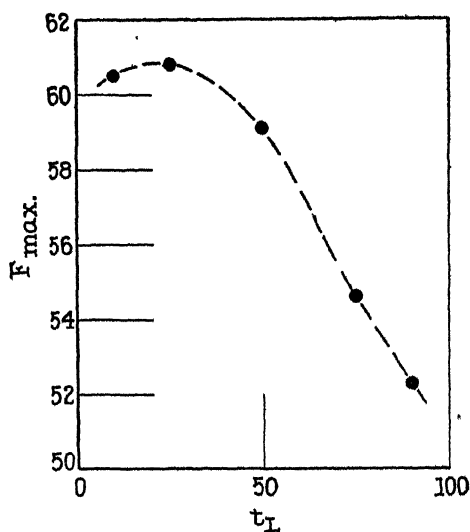
FIG. 14 *a*FIG. 14 *b*

FIG. 14 *a* and *b*. Behavior of $F_{max.}$ as a function of t_L for the data of Figs. 11 and 12. See text.

The influence of light scattered in the eye can be ignored in the present case, we suspect. If it were a significant factor one could scarcely expect to have the symmetrical curves of Figs. 1, 7, and 8.

V

We have now to consider the lower, "rod," sections of the duplex curves in Figs. 7 and 8. In doing so it will be shown that the data using the fragmented field of light provide a significant verification of the method we have used for separating the "rod" and "cone" contributions to the duplex contour. The procedure is to extrapolate the "cone" probability integral back toward $F = 0$. The difference between this and the observed curve is taken to represent the "rod" contribution.

The basis for doing this, and its results, have been described in a number of reports of which some are already cited in this paper. Various lines of evidence have been held to justify the conception that "rod" contributions are pro-

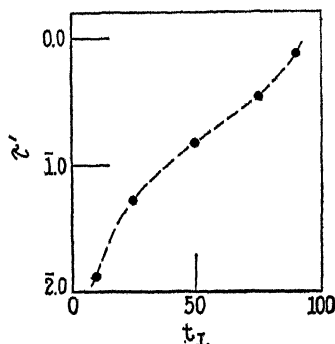


FIG. 15 a

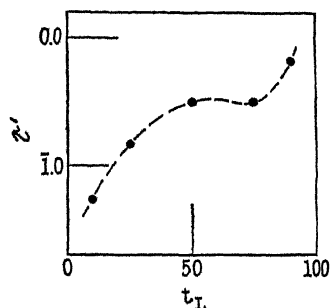


FIG. 15 b

FIG. 15 a and b. The behavior of r' as a function of t_L in the data of Figs. 11 and 12.

gressively inhibited as, with rising F , "cone" effects are increased (*cf.* Crozier and Wolf, 1938-39 a, 1940-41 d, 1941-42 a, etc.). Under most conditions of observation the overlapping of the human "rod" and "cone" curves is complete, so that the measurements in the lower section of the duplex graph represent a summation of "cone" plus uninhibited "rod" effects. Consequently the raw "rod" data do not fall upon a normal probability integral curve. In connection with other visual functions the suggestion has, of course, been made before that at lower intensities one may well be dealing with a combination of rod and cone effects (*cf.* Parsons, 1927). Recently this has been emphasized by Lythgoe (1940). But no attempt seems to have been made to use the idea quantitatively or otherwise than speculatively, except in our analyses of duplex flicker contours.

Figs. 16 and 17 exhibit the calculated extrapolations of the "cone" curves in Figs. 7 and 8 (and Figs. 11 and 12), together with the "difference curves" presumed to depict the "rod" contribution. It is seen that in terms of this analysis the "rod" measurements for $t_L = 0.90$ are almost completely free from

"cone" complication, those at $t_L = 0.75$ only a little less so. For the first time, then, we can be reasonably sure that we are able to deal directly with "rod" flicker observations freed from the inhibitive effects of cone excitation (Crozier

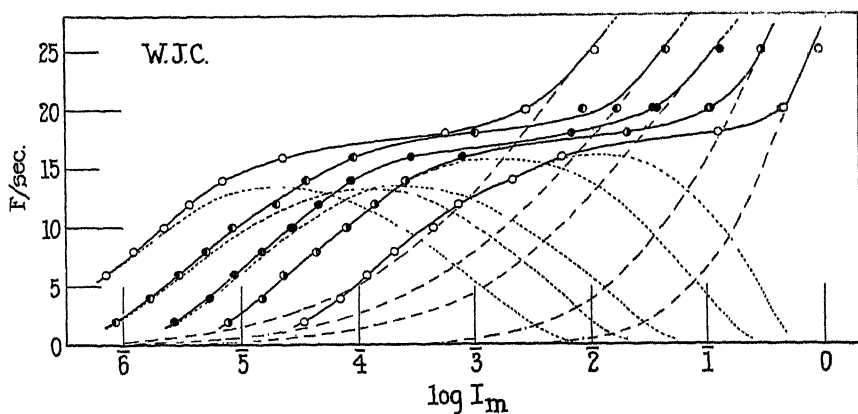


FIG. 16. The lower portions of the graphs in Fig. 7 analyzed by extrapolation of the probability integrals adjusted to the upper "cone" portions. The dotted lines obtained by difference give the "rod" contributions. See text.

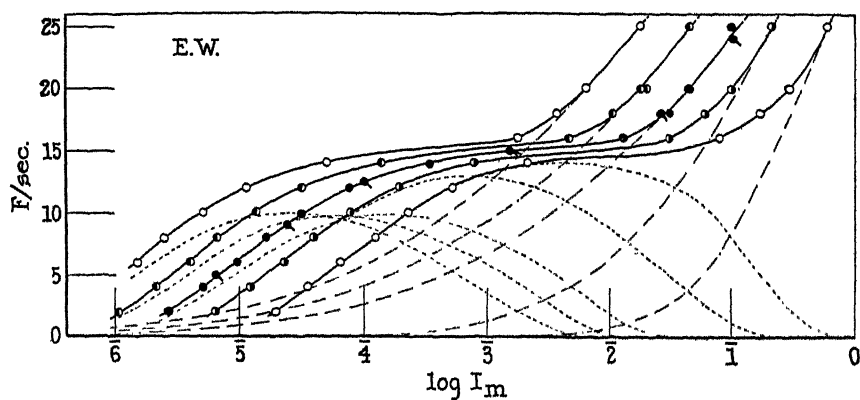


FIG. 17. The lower portions of the graphs in Fig. 8 analyzed by extrapolation of the probability integrals adjusted to the upper "cone" portions. The dotted lines obtained by difference give the "rod" contributions. See text.

and Wolf, 1941-42 c). These measurements as taken directly do adhere well to the probability integral formulation, as shown in Figs. 18 and 19; the others do not.

The rising and the falling branches of the "rod" curves obtained by difference

are shown in Figs. 18 and 19 to adhere very well to probability integrals. The slopes of the rising branches for $t_L = 0.10, 0.25$, and 0.50 are the same. This is what has already been shown for the ordinary data at all values of t_L in the absence of the "pecten" (Crozier and Wolf, 1940-41 *d*). The inhibitive effect of cone excitations must therefore be concerned in these instances with the inhibition of *elements of neural effect* due to excitation of rods, and not to the

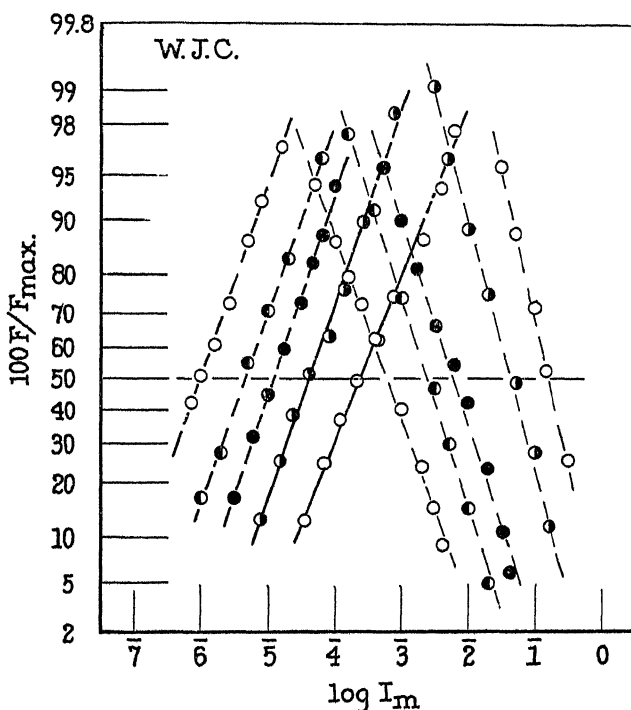


FIG. 18. The rising and declining curves of the rod contributions to the duplex curves of Fig. 7 (as shown in Fig. 16) are here put on a probability grid. Note that for $t_L = 0.75$ and 0.90 the observations are taken directly (solid lines). The other points plotted are read from the dotted lines in Fig. 16.

suppression or complete side-tracking of different numbers of rod *units*. The almost complete elimination of overlap at $t_L = 0.75$ and especially at 0.90 shows that the "rod" $\sigma'_{\log I}$ then becomes quite distinctly greater, and the $F_{max.}$ increases. We know from other lines of evidence that the rod and cone populations of units behave differently with respect to the consequences of an increase in numbers (area): the "cone" $\sigma'_{\log I}$ decreases, the "rod" $\sigma'_{\log I}$ increases when the respective numbers of units are made much larger, regardless of changes in the total number of elements of effect produced, as induced by

changing t_L . We may conclude that when overlapping of the "rod" and "cone" curves is relatively complete a certain number of rod units are forced out of the picture so far as concerns contributing to the discrimination of flicker, while others have their effective contributions reduced.

The *declining* branches of the "rod" curves have slopes which depend, as in other cases, merely on the degree of separation of the "rod" and "cone" popu-

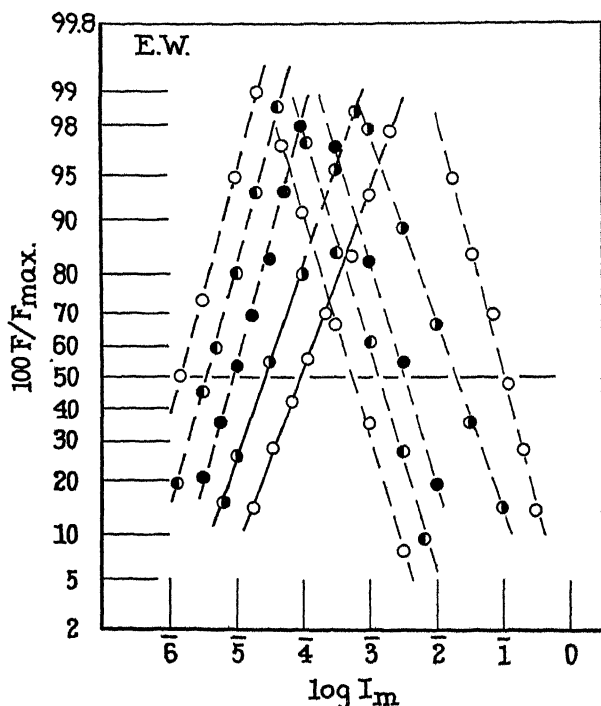


FIG. 19. The rising and declining curves of the rod contributions to the duplex curves of Fig. 8 (as shown in Fig. 17) are here put on a probability grid. Note that for $t_L = 0.75$ and 0.90 the observations are taken directly (solid lines). The other points plotted are read from the dotted lines in Fig. 17.

lations of effects on the $\log I$ axis and on the steepness of the "cone" curves (Crozier and Wolf, 1938-39 *a*, 1940-41 *d*).

It is of some formal consequence that the analysis in Figs. 16 to 19 leads to concordant principles with two sets of curves differing in details of shape and of scale.

The experimental separation of the "rod" and "cone" processes by the use of the grid shadow gives a new kind of proof for the reality of visual duplexity, while emphasizing again the fact that the performance contours do not describe

the physicochemical nature of the primary process of peripheral excitation (*cf.* Crozier and Wolf, 1939-40 *a*, 1940). It is of interest here, and in relation to the summative overlapping of rod and cone effects, to comment upon the character of the subjective end-points. In the measurements of Table III (Fig. 7) the end-point fields at fusion, that is with the flash intensity just below that critical for the appearance of flicker, appear smooth, not granular or "frosted," down to a flash frequency which varies with t_L ; at $t_L = 0.10$, this point is at about $F = 26$; at $t_L = 0.90$, it is at 16. Below these flash frequencies the field is granular down to a low and variable F , below that blue-gray. The grid pattern is clearly visible at $F = 18$ (at $t_L = 0.10$) and down to $F = ca. 1$ (at $t_L = 0.90$). As with the measurements employing colored lights (Crozier and Wolf, 1941-42 *a*, *b*) there is no simple correlation between the subjective character of the end-point fields and the location of singular points on the F log I contour, such as the sharp bend.

We are obliged to Dr. Gertrud Zerrahn-Wolf for her assistance during the course of the experiments.

VI

SUMMARY

1. When there is projected on the retina (man, monocularly) the shadow of a grid which forms a visual field in several distinct pieces (not including the fovea in the present tests), the ordinary properties of the flicker recognition contour (F vs. log I) as a function of the light-time cycle fraction (t_L) can be markedly disturbed. In the present experiments flicker was produced by the rotation of a cylinder with opaque vertical stripes. In the absence of such a grid shadow the "cone" segments of the contours form a set in which F_{max} , and the abscissa of inflection are opposite but rectilinear functions of t_L , while the third parameter of the probability integral ($\sigma'_{\log I}$) remains constant. This is the case also with diverse other animals tested.

In the data with the grid, however, analysis shows that even for low values of t_L (up to 0.50) there occurs an enhancement of the production of elements of neural effect, so that F_{max} rises rather than falls as ordinarily with increase of t_L , although $\sigma'_{\log I}$ stays constant and hence the total number of acting units is presumed not to change. This constitutes valid evidence for neural integration of effects due to the illumination of separated retinal patches. Beginning at $t_L = 0.75$, and at 0.90, the slope of the "cone" curve is sharply increased, and the maximum F is far above its position in the absence of the grid. The decrease of $\sigma'_{\log I}$ (the slope constant) signifies, in terms of other information, an increase in the number of acting cone units. The abscissa of inflection is also much lowered, relatively, whereas without the grid it increases as t_L is made larger. These effects correspond subjectively to the fact that at the end-

point flicker is most pronounced, on the "cone" curve, along the edges of the grid shadow where contrast is particularly evident with the longer light-times.

The "rod" portion of the $F - \log I$ contour is not specifically affected by the presence of the grid shadow. Its form is obtainable at $t_L = 0.90$ free from the influence of summing "cone" contributions, because then almost no overlapping occurs. Analysis shows that when overlapping does occur a certain number of rod units are inhibited by concurrent cone excitation, and that the mean contribution of elements of neural action from each of the non-inhibited units is also reduced to an extent depending on the degree of overlap. The isolated "rod" curve at $t_L = 0.90$ is quite accurately in the form of a probability integral. The data thus give a new experimental proof of the occurrence of two distinct but interlocking populations of visual effects, and experimentally justify the analytical procedures which have been used to separate them.

2. The changing form of the $F - \log I$ contour as a function of t_L , produced in man when the illuminated field is divided into parts by a shadow pattern, is normally found with the bird *Taeniopygia castenotis* (Gould), the zebra finch. The retina has elements of one general structural type (cones), and the $F - \log I$ contour is a simplex symmetrical probability integral. The eye of this bird has a large, complex, and darkly pigmented pecten, which casts a foliated shadow on the retina. The change in form of the $F - \log I$ curve occurs with t_L above 0.50, and at $t_L = 0.90$ is quite extreme. It is more pronounced than the one that is secured in the human data with the particular grid we have used, but there is no doubt that it could be mimicked completely by the use of other grids. The increase of flicker acuity due to the pecten shadow is considerable, when the dark spaces are brief relative to the light. The evidence thus confirms the suggestion (Menner) drawn from comparative natural history that the visual significance of the avian pecten might be to increase the sensory effect of small moving images. It is theoretically important that (as in the human experiment) this may be brought about by an actual decrease of effective retinal area illuminated. It is also significant theoretically that despite the presence of shadows of pecten or of grid, and of the sensory influences thus introduced, the probability integral formulation remains effective.

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FLICKER RESPONSE CONTOURS FOR THE SPARROW, AND THE THEORY OF THE AVIAN PECTEN

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I

For image-forming animals generally it has been found that reduction of the light-time fraction in the light/dark cycle results in enlargement of the F -log I contour for response to flicker and in movement of the curve to *lower* flash intensities.¹ The asymptotic maximum of the curve is directly proportional to the percentage dark-time, and the abscissa of inflection (τ') of the F -log I probability integral is inversely proportional to this quantity; so that τ' and $F_{max.}$ are related rectilinearly. With man, reduction of image area reduces the effect of the light-time fraction on $F_{max.}$, but does not greatly change the dependence of τ' on t_L , if area, wave-length composition, and retinal location are constant. The theoretical bases for these findings have been discussed.¹ This general description applies to the properties of simplex flicker contours and of the photopic ("cone") portions of duplex contours. The scotopic ("rod") segments of duplex contours are the resultants of interaction between "cone" and "rod" neural effects, and the properties of the "rod" $F_{max.}$ as a function of t_L are therefore complex.¹

In birds one has typically to do with a visual system in which there is cast upon the retina the serrated shadow of a large and complexly developed pecten.² Correlated with this we have shown³ that, using the rotating striped cylinder technic,¹ there arises in a bird a novel effect of the light-time fraction upon the form and position of the flicker contour. That the correlation is a causal one is indicated by the fact that homologous changes are induced in the human F -log I contours when, with the same method of producing flicker, an artificial "pecten shadow" is placed on the retina.³ The unique feature of these cases is in the fact that, for bird and man ("cone" curve), the increase of t_L to 0.75–0.90 makes the curve much steeper, increases its $F_{max.}$, and greatly reduces τ' with respect to the values these parameters would have if there were no "pecten effect." The nature of these changes is such as to assist materially in the analytical understanding of the flicker contours, and the effect itself can be shown to have a number of consequences in other directions as well.

¹ *J. Gen. Physiol.*, 1936–37, **20**, 393, 411; 1938–39, **22**, 311, 795; 1939–40, **23**, 531; 1940–41, **24**, 635; 1941–42, **25**, 369.

² Cf. Menner, E., *Zool. Jahrb., Abt. Zool. Physiol.* 1938, **58**, 481.

³ *J. Gen. Physiol.*, 1943–44, **27**, 287.

The diurnal bird used for these particular observations,³ the Australian zebra finch *Taeniopygia*, has in the retina receptor cells of only one general class, histologically. In the tests with human observers the presence of a "pecten shadow" subdividing the illuminated area by several intersecting opaque cross-bars produced no specific effect on the "rod" visual contribution to the flicker contour. The changes found in the "rod" segment of the curves were quantitatively accounted for³ by the neural interactions of rod and cone effects when the cone curve was altered by the "pecten." This gives a further test of the physiological distinctness of the two groups of neural effects in the usually duplex contour for vertebrate visual performance. Physiologically, the simple zebra finch contours have properties homologous with those of the human "cone" curves. Thus, when low light-time fractions are used, where the "pecten effect" does not introduce complications, the relations of the zebra finch flicker contours to wave-length of light are of the same character, qualitatively, as those found for the normal human "cone" curve.⁴ The interesting question thus arises as to whether in general the "rod" curve could be influenced by the "pecten effect;" and if not, why not. It has several further consequences for visual theory, of which we need refer here only to its significance for the analysis of what has often been termed "retinal summation."⁵

The status of certain of these points would be logically more secure if it could be found whether in a bird also the "pecten effect" is one which does not specifically involve the presumptively rod-determined segment of a flicker contour, even when it is naturally apparent in the "cone" segment. Obviously, it is also desirable to determine whether the "pecten effect" is demonstrable at all in more than one kind of bird, even though for purely technical reasons only smaller birds can be used at present for adequate experiments. Data secured in the examination of this point can also be serviceable for theoretical analysis of the visual significance of the difference between (1) "excitable units" and (2) the "elements of neural effect" produced by them, as well as of the neural integration of sensory effects due to excitation of rods and of cones.

The house sparrow, *Passer domesticus* L., is a form taxonomically rather closely near to *Taeniopygia* and has a prominent pecten. Its retina, however, is duplex, containing a fair and easily recognizable proportion of rods.⁶ In keeping with the essential requirements of the duplexity doctrine⁷ we now find that the sparrow's flicker contour is likewise duplex. The curves obtained for *Passer* with different light-time fractions in the flash cycle (white light) are recorded and discussed in the present paper.

³ *J. Gen. Physiol.*, 1941-42, **25**, 381.

⁵ Several following papers.

⁶ Slonaker, J. R., *J. Morphol.*, 1918, **31**, 351; cf. Menner, E., *Z. vergleich. Physiol.*, 1929, **8**, 761; Verrier, M.-L., *Bull. biol. France et Belgique*, 1936, **70**, 197.

⁷ *Proc. Nat. Acad. Sc.*, 1938, **24**, 125, 538; 1939, **25**, 171; *J. Gen. Physiol.*, 1938-39, **22**, 311, 555; 1940-41, **24**, 317, 625.

II

Our sparrows were obtained through the kindness of Dr. F. A. Beach of the American Museum of Natural History, New York City. They had been in captivity for a considerable time, and were the survivors of a large group; mortality of captive sparrows is said to be in excess of 80 per cent. The birds were held for about 4 months before the observations began. From a group of fourteen, six were chosen—three males and three females. They were kept apart from the others, and identified by leg bands; Nos. 1, 5, and 6 were females. They are difficult to catch. Unless the cage is covered with black cloth they fly swiftly against the walls. They did not become tamer with prolonged handling. For each sparrow an observation jar was provided. This was a glass cylinder 5 inches in diameter and 4.5 inches high. The top is a celluloid plate, perforated for ventilation. The bottom is of perforated sheet metal, and on it the bird stands. Sparrows will not sit on a perch in the cylinder (as zebra finches do).

After being put in the cylinders the birds are kept in darkness for at least 2 hours before being tested. This long period in darkness is important to permit the excitement due to handling to subside; without this period of quiescence definite responses to flicker cannot be recognized with any certainty. The procedure was that already described in our experiments with other forms.⁸ After they had quieted down in darkness the sparrows in their cages could be handled without inducing excitement. The cage cylinder is placed within a striped cylinder, which is then rotated at a known, desired speed giving a particular F . The observer being properly dark adapted, the diaphragm admitting light is slowly opened. A sparrow then usually "straightens up" and makes a few head motions as soon as any light is admitted. These motions are easily distinguished from the directed nystagmic motions which ultimately appear as the intensity is increased. If the initial low intensity is maintained briefly, the undirected motions quickly cease and the bird becomes quiescent. When the intensity is increased the end-point for response to flicker is signalized by a typical head nystagmus. This involves turning of the head in a direction following the motion of the stripes on the rotated cylinder, and then a quick return motion. The occurrence of two successive movements of this kind was taken to give the flash intensity I_c , critical for response to flicker. This was necessary because after having made several such movements the birds are liable to intermingle them with undirected motions which could be difficult to evaluate. At lower flash frequencies (and thus at lower intensities) this is not a source of complication; the critical responses are then sharp and simple. Above $F = 25$ the sparrows are more restless; Nos. 3 and 5, and occasionally No. 2, gave responses by hopping around the wall of the cage, following the moving stripes, while the others often stretched out the neck and the whole body as the head showed the typical nystagmus. The forced response to the stripes can be so extreme as to cause mechanical unbalance, so that fast stepping is required to prevent toppling over. The quality of these responses was not changed by altering the light-time ratio, although the decisiveness of the responses, and thus the observer's quickness and certainty in recognizing them, was greater with the decrease of the dark-time

⁸ Cf. *J. Gen. Physiol.*, 1939-40, **23**, 531.

fraction. It should be noted that there is no reflection of this in the variability of the determinations of the mean critical intensities (§ III).

The first series of final measurements was made at $t_L = 0.50$, with 0.90, 0.10, 0.75, and 0.25 following in that order. Of the six individuals used throughout, No. 5 was consistently the most sensitive and No. 6 the least sensitive. The mean rank-order positions of the others fluctuated at random, although No. 1 was often close to No. 6 (all three of these are females). The difference between Nos. 5 and 6 was relatively a little greater for the higher values of t_L , even then amounting to a difference of only 0.04 ± 0.01 log unit but consistent at all values of F . It is curious that this small difference should be detected repeatedly. The order in which the six sparrows were tested was purposely rotated in such a way as to prevent the origin of such differences through systematic errors of observation. Differences of this sort between individuals have been found in other cases (*cf.* footnote 1); where they occur, the averaged data are of course not strictly homogeneous.

III

The data of the present experiment are collected in Table I. The measurements are given as $\log I_m$ for each F and each t_L , together with $\log P.E._I$ for the dispersion of the individual mean determinations (I_1) of the critical flash intensity for the nystagmus end-point.

The scatter of I_1 has been found in all earlier cases⁹ to be such that I_m and σ_I are related rectilinearly, the dispersion of σ_I itself being a constant fraction of mean σ_I at all levels of I_m . It has also been found that with *homogeneous* data¹⁰ the scatter of σ_I for flicker is¹¹ a function of t_L , although the mean values of σ_I are not. With *Taeniopygia*³ it was observed that this effect was not so apparent. This is correlated with the nature of the "pecten effect," which raises $F_{max.}$ at the higher values of t_L , and is found also in the "pecten" experiments with man.¹¹ In the present case $F_{max.}$ changes very little from $t_L = 0.10$ to 0.90, and the character of the inhomogeneity of the data averaged tends to make the scatter of σ_I reverse the usual order since that at $t_L = 0.10$ is greatest (Fig. 1). The mean value of σ_I/I_m is exactly the same as for *Taeniopygia*.⁴

The flicker contours for the sparrow are shown in Fig. 2. It is apparent that they are of the same general form as seen in all tested visually duplex vertebrates.⁷ It is also clear that, as with the zebra finch,³ the shape of the upper (cone) portions of the curves is not independent of the light-time fraction.

The upper parts of the curves in Fig. 2 are shown in Fig. 3 on a probability grid. The lines in Fig. 3 are extended toward $F = 0$ in Fig. 2. By ordinate

⁹ *J. Gen. Physiol.*, 1935-36, **19**, 503; 1936-37, **20**, 211, 363; 1937-38, **21**, 17; 1940-41, **24**, 505, 635; 1941-42, **25**, 89, 293.

¹⁰ *J. Gen. Physiol.*, 1935-36, **19**, 503; *Proc. Nat. Acad. Sc.*, 1937, **23**, 23; 1938, **24**, 130.

¹¹ *J. Gen. Physiol.*, 1940-41, **24**, 635; 1941-42, **25**, 89; 1943-44, **27**, 287.

differences from the curves given by the data at the lower ends the flicker response contributions specifically due to rod excitation are obtained^{12, 13} (dashed lines) in the low intensity region (Fig. 2).

TABLE I

Log I_m and log P.E.₁ for appearance of response to flicker, using white light and light-time fractions 0.10 to 0.90, as a function of flash frequency F , for the sparrow *Passer domesticus*.

Six individuals, the same throughout, for all measurements; three observations on each at every F (i.e., $n = 18$; see text). Plotted in Figs. 1 and 2.

F per sec.	0.10		0.25		0.50		0.75		0.90	
	log I_m	log P.E. ₁	log I_m	log P.E. ₁	log I_m	log P.E. ₁	log I_m	log P.E. ₁	log I_m	log P.E. ₁
2							6.4016	8.5207	6.8000	7.1530
4					6.1338	8.4555	6.5979	8.9807	5.0017	7.9751
									6.9993	7.5538
6					6.2725	8.3497	6.7572	8.7692	5.1358	7.7588
									5.1749	7.6477
8			6.1326	8.3615	6.5009	8.5914	6.9748	7.2399	5.3804	7.9643
10	7.9923	8.4711	6.3646	8.6783	6.7381	8.8686	5.2177	7.5268	5.6050	7.9419
12	6.2240	8.5667								
13			6.7913	8.9547	5.1798	7.6276	5.6567	7.6323	4.0454	6.2887
14	6.5999	8.6401								
15			5.1929	7.5612	5.5708	6.1737	4.0286	6.5862	4.4150	6.8984
					5.5542	7.6726				
16	5.0111	7.4228								
17			5.6656	7.8011	4.0481	6.2136	4.4972	6.8539	4.9050	6.9326
18	5.5097	7.5064	5.9208	6.1544	4.2936	6.7446	4.6907	6.8405	3.0910	5.5594
20	5.8316	6.2107	4.2615	6.8967	4.6233	6.9924	4.9435	5.2613	3.3214	5.9334
	5.8398	6.0691			4.6214	5.2613			3.2991	4.0968
25	4.2415	6.7299	4.6698	5.1074	3.0378	5.4288	3.3286	5.8668	3.5947	4.0522
30	4.5667	6.9968	4.9889	5.4293	3.3657	4.1058	3.5718	4.0202	3.7893	4.2301
35	4.9126	5.3867	3.3379	5.5862	3.6973	4.7401	3.8094	4.1651	3.9548	4.3792
					3.7252	5.8959				
40	3.3185	5.8011	3.7332	4.1115	2.0846	4.1521	2.0257	4.2817	2.1159	3.0703
45	3.7728	4.0708	2.1711	4.6416	2.5013	4.9271	2.2485	4.6683	2.3383	4.7397
									2.3731	3.0393
47					2.7483	4.7646				
50	2.4420	4.6880	2.8561	3.3238	1.2953	3.5612	2.7017	3.1042	2.8047	3.3196
53	1.0888	3.5931	1.5620	2.1273	1.9483	2.2074	1.2036	3.6115	1.2918	3.9736
54									1.5020	2.0012
55	0.1670	2.5775	0.5488	1.2463	0.9516	1.1421	0.2512	2.7133	1.7884	2.2140
									0.3460	1.0564

We have pointed out⁸ that the changed shape of the F -log I ("cone") curve at $t_L = 0.75$ and 0.90 can be understood as due to the activation of a larger

¹² *J. Gen. Physiol.*, 1936-37, 20, 203; 1940-41, 24, 635; etc.

¹³ *J. Gen. Physiol.*, 1941-42, 25, 293, 369.

number of cone units than would be concerned in critical recognition of flicker if the pecten shadow were not present. At the same time (since F_{max} does

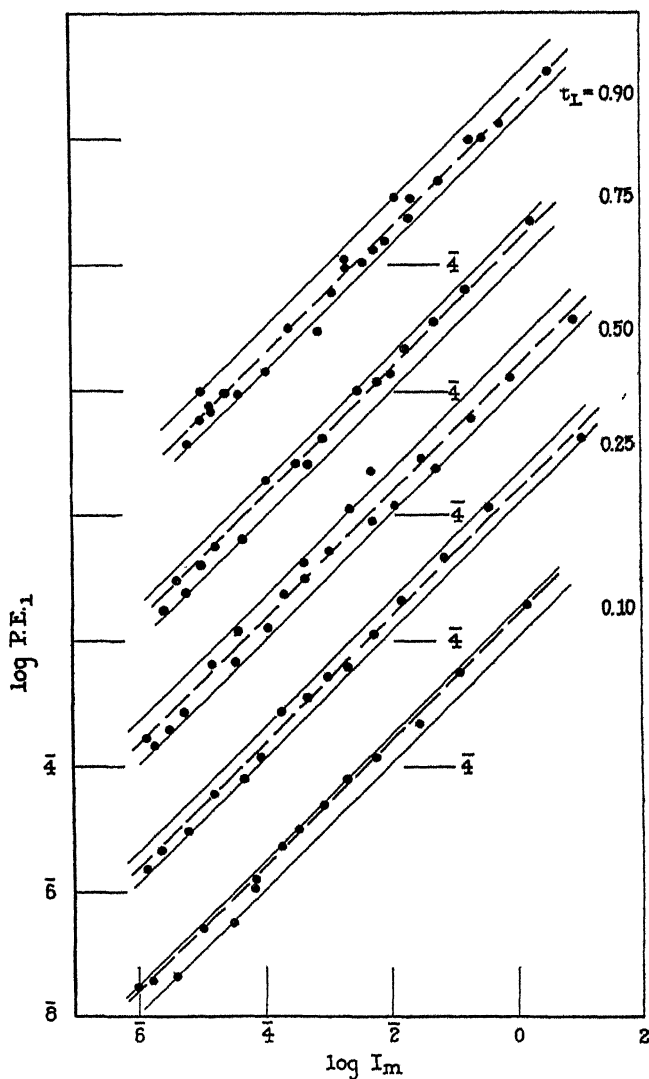


FIG. 1. Variation of critical intensities for response to flicker at different light-time fractions; data in Table I. The mean proportionality constants are the same for the different light-time fractions, the curves being separated arbitrarily for convenience.

not greatly change) the average number of elements of neural effect derived from each cone unit must be reduced. When no "pecten effect" is involved,

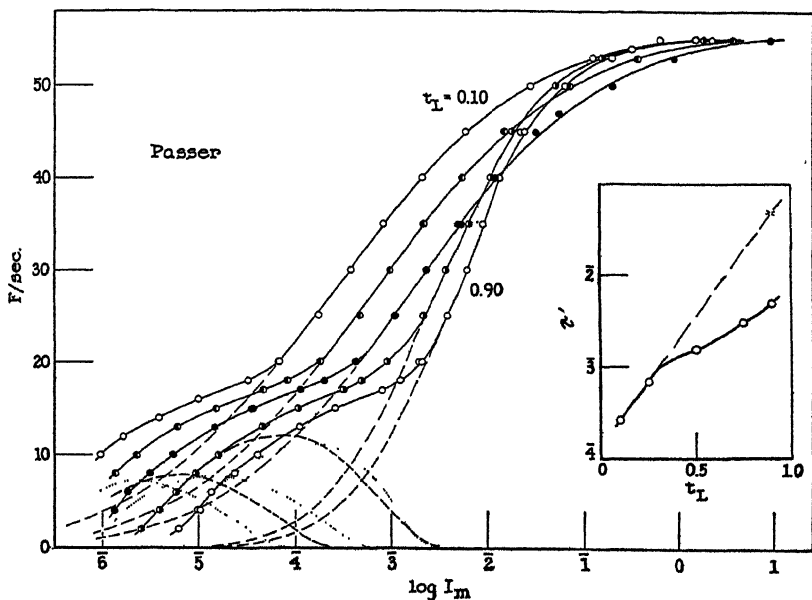


FIG. 2. Flicker response contours for the house sparrow. Data in Table I. The photopic curves are the probability integrals shown in Fig. 3. The analyses of the lower, scotopic sections are discussed in the text: the ascending and descending "rod" curves here shown dashed are shown on a probability grid in Fig. 4. The inset shows the actual relationship between abscissa of inflection of the "cone" curves (r') as a function of t_L , the extrapolation indicating the expected course of this relationship were there no effect due to the pecten; see text.

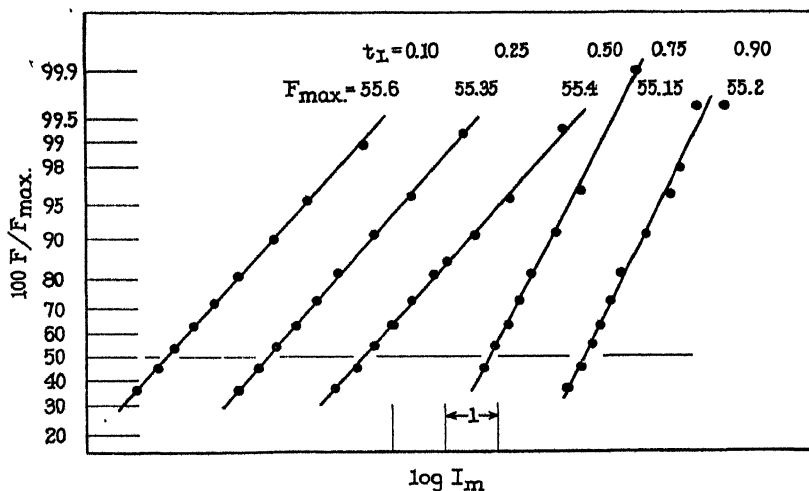


FIG. 3. The data of Fig. 2, photopic segments, shown on a probability grid. The lateral separation of the graphs is arbitrary, for convenience. The maximum F to which each curve is computed is shown.

with its accentuation of visual contrast at the shadow edges, increasing t_L does not change the slope constant of the contour ($\sigma'_{\log I}$), but F_{max} gets lower; in Fig. 3 the slight differences in slope in $t_L = 0.10, 0.25, 0.50$ are probably not significant, although Fig. 2 (inset) does show that at $t_L = 0.50$ the "pecten effect" is already detectable. This is taken to mean that the same number of units is concerned, with reduction in the effective contribution from each.¹

We can gain an idea of the effect due to the presence of the pecten if we try to construct the appearance of the set of flicker contours in Fig. 2 on the basis of expectation if the pecten were not present. In various cases of this kind¹ we know that the abscissa of inflection of the photopic flicker contour (τ') is a rectilinear function of t_L ; in *Taeniopygia*³ this is also true, as in our human tests with a "pecten shadow," up through $t_L = 0.50$. With the sparrow the effect is in one sense not so extreme as in the zebra finch (*cf.* footnote 3), but is already apparent at $t_L = 0.50$. As the inset in Fig. 2 shows, the rate of increase of τ' with increase of t_L falls off after $t_L = 0.25$. Extrapolating to $t_L = 0.90$, on the assumption justified by the data on forms without pecten,¹ we would expect τ' for the sparrow at $t_L = 0.90$ to be at about 2.68—a whole log unit above its actual value as found. The gain in acuity for the recognition of moving stripes, under these conditions, is thus a little less than found for the zebra finch, and about the same as produced in man by the particular "artificial pecten" used in our experiments.³

This gain in acuity is by no means inconsiderable, and is perhaps even more striking because the low t_L contours for the sparrow, although of just about the same F_{max} , as those for the zebra finch, are situated at flash intensities about 0.4 log unit lower. In more usual terms it corresponds to a raising of the critical flash frequency, due to the pecten, from 27.5 per second to 49 per second at $t_L = 0.90$ and $\log I_m (\text{ml.}) = 2.68$. The "pecten effect" has also another consequence. At high flash intensities the acuity for moving dark stripes is made much more nearly the same for all light-time fractions. Instead of covering a span of about 2.3 log I units, the curves spread only by 0.85 log unit, and the 0.75 and 0.90 contours approach that for $t_L = 0.10$. To what extent this effect is exhibited for *single* moving images we do not as yet know, but qualitatively it is fairly certain that it must exist. The avian pecten is in effect an internal device for producing visual flicker—a moving image, if small enough, moves (as it were) across a fence of opaque pickets.

IV

We have discussed the "pecten effect" as exhibited in the photopic segment of the duplex sparrow flicker contour. It is of the same kind as that found in the simplex F -log I curves for the zebra finch, and for man. We now consider the modifications produced in the scotopic "rod" curves. In our analysis of the "rod" curves for man, with the "artificial pecten," it was pointed out³ that

the raw "rod" data suffered no such gross changes of slope as found with the "cone" curves. The actual "rod" contributions as dissected out after extrapolation of the "cone" probability integrals¹² gave every evidence of suffering from partial inhibition due to concurrent excitation of cones, rather than being directly influenced by the "pecten" shadow.^{3, 13}

The extracted "rod" curves in Fig. 2 are put upon a probability grid in Fig. 4. The properties of this set of curves are qualitatively the same as those of the

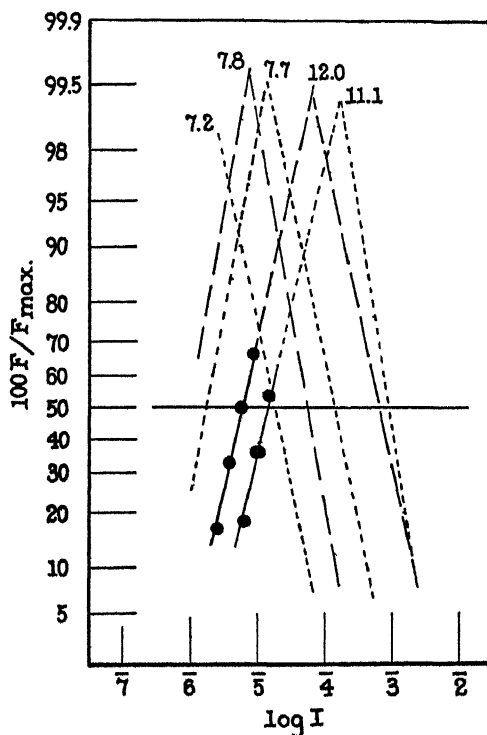


FIG. 4. The computed rising and falling "rod" contribution curves shown in Fig. 2 are here put upon a probability grid. See text.

corresponding sets we have analyzed for human observers.³ When, for $t_L = 0.75$ and 0.90 , a portion of the curve of observations escapes complication by overlapping cone excitation the data then fall on the probability integral required by the subtraction of the "cone" ordinates for the remainder of the scotopic segment. Again, as shown, the slope constant ($\sigma'_{\log I}$) for these ascending curves is less than for those in which the "rod" and "cone" contributions overlap completely (e.g., for $t_L = 0.25, 0.50$). This finding we have already indicated to be the expected one when the number of effective rod units

is increased³ (*i.e.*, the reverse of the situation with cone units). Since the "rod" F_{max} , also increases sharply, the possibility is not excluded that there may indeed be a true effect of the pecten on the production of action elements from rod units, although we think this unlikely. Even in the absence of a "pecten shadow" the "rod" F_{max} , increases a little (man) with increase of t_L ,¹⁴ as an automatic consequence of the integration of "rod" and "cone" effects when the "cone" τ' is increased faster than the "rod" τ' by enlarging t_L . The inhibition of both "rod" units and elements of effect due to "cone" excitations is sufficient to account for the behavior of the "rod" F_{max} , in Figs. 2 and 4. We therefore concluded that there is no evidence of a direct influence of the pecten on the rod flicker response function.

This question can be investigated more directly with man. We have pointed out¹³ that by using lights of limited wave-length composition (*e.g.*, blue) rather than white, image areas and locations can be found in which there is essentially no overlapping of the rising scotopic and the photopic flicker contours. The subdivision of such an image by a "pecten shadow" should permit analysis of the rod curves without admixture of cone effects.

V

SUMMARY

The flicker contour for the house sparrow *Passer domesticus* is duplex, corresponding to the presence of both rods and cones in the retina. The presence of the pecten brings about changes in the "cone" part of the contour when the light-time in the flash cycle is varied. These changes are of the same sort as those we have already described for the visually simplex zebra finch, and for man provided with an artificial "pecten shadow." The changes are such as to greatly enhance flicker acuity for small dark-times (moving stripe technique). The form of the scotopic part of the duplex contour (also as in the case with man) gives no evidence that rod excitation is specifically influenced by the presence of the pecten. The changing integration of "rod" and "cone" effects as the light-time fraction is altered provides another means of testing the theory used for the analytical separation of the two components of the duplex flicker contour.

¹⁴ *J. Gen. Physiol.*, 1940-41, 24, 635.

THE EFFECT OF CARBON DIOXIDE TENSION ON THE METABOLISM OF CEREBRAL CORTEX AND MEDULLA OBLONGATA*

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I

Warburg, Posener, and Negelein (1924) observed an increase in anaerobic glycolysis of sarcoma when carbon dioxide tension and bicarbonate concentration were raised at constant pH. A fivefold increase in the carbon dioxide tension and bicarbonate concentration was found to double both oxygen uptake and aerobic glycolysis by rat retina (Craig and Beecher, 1943 c). In the present study the observations have been extended to cerebral cortex and medulla oblongata from the cat. Some measurements at low oxygen tensions with bicarbonate medium have been included to complement data obtained with phosphate medium (Craig and Beecher, 1943 a).

II

Fifty-three unselected cats were employed. Following painless sacrifice, a sagittal cut was made through the head with a guillotine, to permit immediate exposure of the brain. At room temperature (about 25° C.) slices were cut from the outer surface of the cerebral cortex, or from the medulla oblongata in a parasagittal plane. The phosphate medium was made up to contain 0.119 M NaCl, 0.0026 M KCl, 0.0019 M CaCl₂, 0.00072 M MgCl₂, 0.0033 M NaH₂PO₄, 0.0185 M Na₂HPO₄, and 0.011 M dextrose at pH 7.4. In this medium oxygen uptake was determined by the first method of Warburg (1923). The bicarbonate medium was made up to contain 0.0024 M KCl, 0.0017 M CaCl₂, 0.00066 M MgCl₂, and 0.011 dextrose. The sum of NaCl and NaHCO₃ concentration was 0.140 M.

The concentration of these anions, the carbon dioxide tension, and the calculated pH are given in the tables. In this medium oxygen uptake and total acid output were determined by the second method of Warburg (1924). The lactic acid that accumulated in the vessels was determined colorimetrically by the method of Barker and Summerson (1941).¹

It was observed that more of a precipitate formed in phosphate medium than in bicarbonate medium with 5 per cent carbon dioxide. Although the media contained

* Aided by a grant from the Milton Fund of Harvard University to Henry K. Beecher.

¹ The analyses were carried out by Miss Ann Murphy.

about 0.002 M CaCl_2 , analyses by the method of Fiske and Logan (1931)¹ indicated that calcium remaining in solution was only 0.0002 M in phosphate medium at 7.4 and in medium containing 0.100 M NaHCO_3 in equilibrium with 20 per cent carbon dioxide; with 0.025 M NaHCO_3 and 5 per cent carbon dioxide, 0.0011 M remained in solution. In order to maintain a uniform calcium concentration in solution some of the bicarbonate media were made up to contain only 0.0002 M CaCl_2 .

The gases were mixed in 20 liter carboys over saturated CaCl_2 solution and analyzed on the Haldane apparatus.¹ The temperature of the water bath was 38°. The shaker speed was 120 cycles per minute. At the conclusion of the manometric observations the tissue slices were removed from the vessels and dried to constant weight.

The following symbols are defined:—

Q_{O_2} = oxygen uptake in c. mm./hr./mg. tissue.

Q_A = total carbon dioxide output in c. mm./hr./mg. tissue, including respiratory carbon dioxide and carbon dioxide displaced from the bicarbonate buffer by lactic acid.

Q_G = $Q_A - Q_{O_2}$ = carbon dioxide output due to lactic acid in c. mm./mg./hr.; 1 c. mm. is equivalent to 0.004 mg. of lactic acid. Q_{O_2} is assumed to be numerically equal to respiratory carbon dioxide.

L. A. = total lactic acid formed in preliminary period of equilibration (10 to 15 minutes) and experimental period of 2 hours, in mg./gm. tissue.

The standard error of the mean for small series, is given for each set of data:

$$\sqrt{\frac{\sum d^2}{n(n-1)}}$$

III

Time.—In contrast to the results obtained previously with phosphate medium (Table IV) when there was no change in Q_{O_2} from the 1st to the 2nd hour, the Q_{O_2} for both cortex and medulla measured in bicarbonate medium decreased significantly in the 2nd hour (Table I).² In the 2nd, 3rd, and 4th hours, however, the Q_{O_2} was nearly constant. The decrease from the 1st to the 2nd hour was observed with both high and low concentrations of calcium (Table III). With cortex the Q_G was negligible in the 2nd hour and became negative in the

² In retina the Q_{O_2} did not decline in the 2nd hour except in the low carbon dioxide bicarbonate medium.

Oxygen uptake of rat retina

Medium	No. of observations	1st hour Q_{O_2}	2nd hour Q_{O_2} as per cent of 1st hour	Source of data
Phosphate	10	17.9 \pm 0.60	95 \pm 3.7	Craig and Beecher, 1943b
Bicarbonate 5 per cent CO_2	10	31.0 \pm 2.3	98 \pm 6.7	Craig and Beecher, 1943c
Bicarbonate 1 per cent CO_2	15	16.0 \pm 2.0	73 \pm 5.7	Craig and Beecher, 1943c

TABLE I

*Variations with Time in Bicarbonate Medium*95 per cent O₂, 5 per cent CO₂, 0.025 M NaHCO₃, 0.0011 M Ca in solution. Group A.QO₂ = oxygen uptake in c. mm./hr./mg. tissue (dry).Q_G = Q_A - QO₂, where Q_A = total carbon dioxide output in c. mm./hr./mg. tissue (dry) including respiratory carbon dioxide and carbon dioxide set free by glycolysis. 0.004 mg. lactic acid would set free 1.0 c. mm. of carbon dioxide.

Time	No. of cats No. of observations	QO ₂	Q _G
Cortex			
<i>hrs.</i>			
1	15/15	11.8 ± 0.41	4.0 ± 0.27
2	15/15	8.6 ± 0.38	0.2 ± 0.26
3	8/8	8.9 ± 0.49	-0.8 ± 0.30
4	5/5	8.0 ± 0.66	-0.9 ± 0.28
Medulla			
1	14/14	4.0 ± 0.30	2.1 ± 0.23
2	14/14	3.1 ± 0.16	1.0 ± 0.05
3	7/7	2.9 ± 0.20	0.4 ± 0.18
4	3/3	2.7 ± 0.21	0.6 ± 0.10

TABLE II

*Variations with Time in Phosphate Medium**

A. Lactic acid output in phosphate medium by cerebral cortex

Soluble calcium, 0.0002 M				
Cat No.	Output after preliminary period plus 1 hr.	Length of additional period	Total output including additional period	Mean Q _G for additional period
	<i>mg./gm.</i>	<i>hrs.</i>	<i>mg./gm.</i>	
416	51	4	82	1.9
417	59	6	143	3.5
418	76	3	114	3.2
419	62†	2	94	4.0
420	61	3	104	3.6
421	77	3	109	2.7
422	68	3	105	3.1
Mean				3.1 ± 0.25

B. Oxygen uptake for the animals listed in A

Time	QO ₂
<i>hrs.</i>	
1	10.9 ± 0.55
2	10.5 ± 0.20
3	9.8 ± 0.16
4	9.1 ± 0.18

* The animals referred to in this table were members of another series for which further data have been reported elsewhere (Craig, 1943).

† Preliminary period plus 2 hours.

TABLE III

Variations in Metabolism with the Concentration of Calcium Remaining in Solution in Bicarbonate Medium

L.A. = lactic acid in mg./gm. tissue, determined colorimetrically, formed in 2 hours plus preliminary period.

Other symbols as defined in Table I.

Group	B	C	D	A	E	F
Ca ⁺⁺ M.....	0.0002	0.0020	0.0002	0.0011	0.0020	0.0016
pH.....	7.48	7.48	7.48	7.48	6.78	6.88
O ₂ , vol. per cent. . .	99	99	95	95	95	80
CO ₂ , vol. per cent. .	1	1	5	5	5	20
NaHCO ₃ , M.....	0.005	0.005	0.025	0.025	0.005	0.025
NaCl, M.....	0.135	0.135	0.115	0.115	0.135	0.115

Cortex

No. of cats							
No. of observations		3/7	4/4	3/8	15/15	2/2	2/2
QO ₂	1st hr.	14.4 ±0.51	10.8 ±0.45	15.5 ±0.32	11.8 ±0.41	8.7 ±0.5	11.2 ±0.6
	2nd hr.	11.3 ±0.26	7.0 ±1.36	12.2 ±0.24	8.6 ±0.38	8.6 ±0.7	8.2 ±0.2
Q _G	1st hr.	4.0 ±0.35	1.7 ±0.81	8.9 ±0.26	4.0 ±0.27	0.4 ±0.1	3.2 ±0.2
	2nd hr.	1.2 ±0.13	-0.4 ±0.75	4.2 ±0.26	0.2 ±0.26	-0.7 ±0.1	-0.4 ±0.3
No. of cats							
No. of observations		3/14	4/8	3/18	7/14	2/4	2/4
L.A.		43 ±1.1	10 ±2.8	58 ±1.3	31 ±4.8	7 ±6.5	23 ±2.1

Medulla

No. of cats							
No. of observations		3/7	3/3	3/9	14/14	2/2	2/2
QO ₂	1st hr.	4.4 ±0.53	4.3 ±0.93	5.2 ±0.41	4.0 ±0.30	2.8 ±0.5	3.5 ±1.0
	2nd hr.	2.8 ±0.28	3.2 ±0.51	3.4 ±0.15	3.1 ±0.16	2.9 ±0.1	2.0 ±0.4
Q _G	1st hr.	1.5 ±0.24	1.4 ±0.58	3.2 ±0.32	2.1 ±0.23	0.0 ±0.3	2.3 ±0.5
	2nd hr.	0.5 ±0.18	0.5 ±0.29	1.2 ±0.10	1.0 ±0.05	-0.2 ±0.2	0.6 ±0.2
No. of cats							
No. of observations		3/14	3/6	3/18	7/14	2/4	2/4
L.A.		13 ±2.3	6 ±1.4	22 ±1.2	15 ±1.8	8 ±0.8	5 ±1.8

3rd and 4th hours. This could indicate utilization of lactate (Dixon, 1935). With medulla, the Q_G declined with time but was always positive. One would like to know whether aerobic glycolysis is a normal feature of the metabolism of medulla *in vivo*, as it is of tumors. With cortex in phosphate medium the lactic acid remaining in the vessel was greater at the end of 4 hours than 1 hour (Table II). The mean Q_G was 3.1 for the last 3 hours.

Calcium.—The effects of cation imbalance in the medium on the metabolism of cerebral cortex have been described by Ashford and Dixon (1935), Dickens and Greville (1935), and Canzanelli, Rogers, and Rapport (1942). One characteristic accompaniment of cation imbalance in the shape of a low calcium concentration, namely, an increase in both Q_{O_2} and Q_G , is shown in Table III at two levels of carbon dioxide-bicarbonate concentration. In medulla, the effects of changes in calcium concentration were less pronounced than in cortex.

The calcium concentration may also account for the differences in glycolysis between phosphate and bicarbonate media (Table IV). Aerobic glycolysis was higher in phosphate medium than in bicarbonate medium (AI) but the reverse was true for anaerobic glycolysis (HK). Ashford (1934) found anaerobic glycolysis by brain slices to be 60 per cent greater in bicarbonate medium than in phosphate. In the case of anaerobic glycolysis both calcium concentration and carbon dioxide tension may contribute to the difference between the two buffers. In the case of aerobic glycolysis, data for media containing the same amount of calcium in solution are available. For phosphate medium (Beecher and Craig, 1943, Table V), and bicarbonate media containing 1 per cent and 5 per cent carbon dioxide (B and D) the Q_G for the 1st hour was 7.4, 4.0, and 8.9 respectively. The fact that Q_G was lower with 1 per cent carbon dioxide than with phosphate indicates that phosphate may have an influence on glycolysis independent of calcium concentration and carbon dioxide tension.

Low Oxygen Tension.—The double vessel method determines the total amount of acid produced in metabolism including carbon dioxide and lactic acid. The data obtained by this method (Table IV) revealed an unexpected feature of the metabolism of medulla. When the oxygen content was diminished from 95 to 3 volumes per cent, the mean Q_A decreased from 5.1 to 3.8 although the mean Q_G increased 60 per cent. In cortex the decrease in carbon dioxide from respiration was balanced by the increase in glycolysis. The results in Table IV further emphasize the point brought out by the experiments with phosphate medium, that glycolysis in medulla is much smaller than in cortex and less sensitive to changes in oxygen tension. The decrease in lactic acid output by medulla in phosphate medium when the oxygen was diminished from 3 to 0.5 volumes per cent did not appear in bicarbonate medium.

In both tissues Q_G decreased from the 1st to the 2nd hour. It was noted that in each tissue the decrease in absolute units, $Q_{G(1-2)}$, was independent of oxygen tension.

TABLE IV

Low Oxygen Tension

Bicarbonate medium with 0.0011 M Ca in solution; phosphate medium with 0.002 M Ca in solution. Phosphate data from Craig and Beecher (1943a).

$Q_{G(1-2)}$ represents the decrease in Q_G from the 1st to the 2nd hour.

Other symbols as in Tables I and III.

		Bicarbonate medium			Phosphate medium		
Group.....		A	G	H	I	J	K
O_2 , vol. per cent....		95	3	0.5	100	3*	0.5

Cortex							
No. of cats		15/15	6/7	7/7	15/15	9/9	9/9
No. of observations							
Q_{O_2}	1st hr.	11.8 \pm 0.41	2.5 \pm 0.72	0.2 \pm 0.73	10.5 \pm 0.35	2.0 \pm 0.11	0.1 \pm 0.12
	2nd hr.	8.6 \pm 0.38	1.3 \pm 0.63	0.6 \pm 0.80	10.2 \pm 0.27	2.4 \pm 0.10	0.7 \pm 0.13
	Mean.....	10.2 \pm 0.35	2.0 \pm 0.42	0.3 \pm 0.57	10.4 \pm 0.31	2.2 \pm 0.11	0.4 \pm 0.07
Q_A mean.....		12.3 \pm 0.43	13.5 \pm 0.71	15.5 \pm 1.62			
Q_G	1st hr.	4.0 \pm 0.27	13.4 \pm 0.66	16.7 \pm 1.54			
	2nd hr.	0.2 \pm 0.26	9.4 \pm 0.55	13.6 \pm 1.29			
	(1-2)	3.8 \pm 0.32	3.9 \pm 0.67	3.2 \pm 0.60			
No. of cats		7/14	6/14	7/14	14/14	7/7	7/7
No. of observations							
L.A.....		31 \pm 4.8	145 \pm 7.1	170 \pm 7.7	52 \pm 3.9	121 \pm 12.7	138 \pm 14.5

Medulla							
No. of cats		14/14	7/7	7/7	14/14	7/7	7/7
No. of observations							
Q_{O_2}	1st hr.	4.0 \pm 0.30	1.6 \pm 0.43	0.4 \pm 0.47	3.5 \pm 0.18	0.9 \pm 0.14	0.2 \pm 0.09
	2nd hr.	3.1 \pm 0.16	1.0 \pm 0.34	0.0 \pm 0.03	3.4 \pm 0.18	1.0 \pm 0.14	0.4 \pm 0.08
	Mean.....	3.5 \pm 0.19	1.3 \pm 0.39	0.2 \pm 0.33	3.5 \pm 0.17	1.0 \pm 0.14	0.3 \pm 0.08
Q_A mean		5.1 \pm 0.27	3.8 \pm 0.50	2.6 \pm 0.52			
Q_G	1st hr.	2.1 \pm 0.23	3.0 \pm 0.19	2.8 \pm 0.33			
	2nd hr.	1.0 \pm 0.05	2.0 \pm 0.10	1.9 \pm 0.13			
	(1-2)	1.1 \pm 0.23	1.0 \pm 0.14	0.9 \pm 0.27			
No. of cats		7/14	7/14	7/14	14/14	7/7	8/8
No. of observations							
L.A.....		15 \pm 1.8	23 \pm 2.2	25 \pm 1.5	18 \pm 2.2	32 \pm 3.1	23 \pm 2.2

* The oxygen mixture used with medulla in this group averaged 2 volumes per cent instead of 3.

pH.—The oxygen uptake of cerebral cortex in neutralized serum without carbon dioxide has been studied over a wide range of pH by Canzanelli, Greenblatt, Rogers, and Rapport (1939). They noticed little change between pH 7.4 and pH 8.3, but at pH 9 there was a 30 per cent elevation in Q_{O_2} . In the present study measurements were made in both phosphate and bicarbonate medium at pH 6.8, 7.4, and 8.2. In phosphate medium (Table V) Q_{O_2} was little altered in this range, but L.A. increased with pH, particularly in cortex.

TABLE V
Hydrogen Ion Concentration in Phosphate Medium
Oxygen, 100 per cent. NaCl, 0.119 M. Symbols as in Tables I and III

Group.....		L	I	M
pH.....		6.8	7.4	8.2
NaH ₂ PO ₄ , M.....		0.0109	0.0033	0.00022
Na ₂ HPO ₄ , M.....		0.0109	0.0185	0.0216
Ca ⁺⁺ , M.....		0.0008	0.0002	0.0002
Cortex				
No. of cats		2/2	15/15	3/5
No. of observations				
QO ₂	1st hr.	9.8 ±0.9	10.5 ±0.35	10.9 ±0.54
	2nd hr.	9.4 ±0.3	10.2 ±0.27	10.4 ±0.54
L.A.		36 ±0	52 ±3.9	83 ±5.0
Medulla				
No. of cats		2/2	14/14	3/5
No. of observations				
QO ₂	1st hr.	3.6 ±0.2	3.5 ±0.18	3.9 ±0.32
	2nd hr.	3.5 ±0.3	3.4 ±0.18	4.1 ±0.36
L.A.		18 ±4	18 ±2.2	25 ±3.1

In bicarbonate medium (Tables III and VI) an attempt was made to separate the effects of acidity from those of carbon dioxide and of bicarbonate; changes in pH between seven pairs of buffers were studied. Changes in Q_{O_2} followed changes in carbon dioxide tension in pairs CE and NP, and in bicarbonate concentration in pairs AE and DP. Changes in Q_G or L.A. followed changes in carbon dioxide tension in pairs CE and AF and in bicarbonate concentration in pairs AE, DP, and BO. Glycolysis appeared to be much more sensitive

to bicarbonate than to carbon dioxide in these instances. From a comparison of BO and DO with DP and NP it would appear that pH was secondary in im-

TABLE VI
Carbon Dioxide Tension

Medium containing 0.0002 M Ca in solution. Symbols as in Tables I and III					
Group	B	D	N	O	P
pH.....	7.48	7.48	7.48	8.18	8.08
O ₂ , vol. per cent....	99	95	80	99	95
CO ₂ , vol. per cent....	1	5	20	1	5
NaHCO ₃ , M.....	0.005	0.025	0.100	0.025	0.100
NaCl, M.....	0.135	0.115	0.040	0.115	0.040

Cortex					
No. of cats No. of observations		3/7	3/8	4/4	3/5
Q _{O₂}	1st hr.	14.4 ±0.51	15.5 ±0.32	13.6 ±1.89	16.9 ±1.24
	2nd hr.	11.3 ±0.26	12.2 ±0.24	10.6 ±1.54	10.9 ±0.35
Q _a	1st hr.	4.0 ±0.35	8.9 ±0.26	8.6 ±2.41	6.3 ±0.52
	2nd hr.	1.2 ±0.13	4.2 ±0.26	2.2 ±1.58	0.9 ±0.21
No. of cats No. of observations		3/14	3/18	4/8	3/10
L.A.		43 ±1.1	58 ±1.3	99 ±16.6	62 ±3.7
		119 ±9.6			

Medulla					
No. of cats No. of observations		3/7	3/9	5/9	3/5
Q _{O₂}	1st hr.	4.4 ±0.53	5.2 ±0.41	3.1 ±0.45	7.0 ±0.71
	2nd hr.	2.8 ±0.28	3.4 ±0.15	2.4 ±0.31	5.1 ±0.61
Q _a	1st hr.	1.5 ±0.24	3.2 ±0.32	3.3 ±0.47	2.1 ±0.28
	2nd hr.	0.5 ±0.18	1.2 ±0.10	1.9 ±0.40	1.3 ±0.17
No. of cats No. of observations		3/14	3/18	5/18	3/10
L.A.		13 ±2.3	22 ±1.2	27 ±2.5	23 ±2.2
		30 ±2.4			

portance to the composition of the buffer mixture. However, variations in pH in phosphate medium had considerable effect on glycolysis; therefore, the results

obtained with a constant ratio of carbon dioxide to bicarbonate would seem to be more significant concerning the effect of carbon dioxide tension.

The question of the relative penetration of carbon dioxide and bicarbonate arises here. Jacobs (1920) attributed the acid taste of a $M/2$ solution of NaHCO_3 at pH 7.4 to the more rapid penetration of carbon dioxide into the cell. Working with longer exposure time, more comparable with the present one, Smith and Clowes (1924) studied the influence of carbon dioxide on the velocity of division of marine eggs. They observed that while carbon dioxide decreased the velocity, the effectiveness of carbon dioxide declined as the concentration of bicarbonate increased. They hypothesized penetration of bicarbonate into the cell in simple proportion to the extracellular concentration. In discussing the experiment with carbon dioxide and retina (Craig and Beecher, 1943 c) it was argued that bicarbonate must enter the cell in proportion to carbon dioxide when the concentration of the buffer mixture was increased in order to prevent the cells from turning acid, and consequently forming lactic acid at a lower rate than was observed. Because of the sensitivity of glycolysis to pH (Table V), the same argument applies to the data for cortex and medulla. The reservation should be made, however, that the intracellular pH may be controlled by buffers other than bicarbonate.

Carbon Dioxide-Bicarbonate.—The effects of this variable depended very much upon the conditions under which it was studied. With media containing cations as nearly in balance as possible with regard to calcium, namely 0.0020 M (C) and 0.0011 M (A), both at pH 7.48 (Table III), the results were much the same for cortex and medulla. The Q_{O_2} was not affected by the change in carbon dioxide from 1 to 5 per cent, but Q_g and L.A. increased.

With the calcium content constant, but at a lower level (0.0002 M), an increase in buffered carbon dioxide from 1 to 5 per cent (BD) again had no significant effect on Q_{O_2} . Q_g , however, doubled in both tissues and L.A. was increased significantly. A further increase in carbon dioxide to 20 per cent (DN), when bicarbonate became the principle anion, brought about a significant decrease in Q_{O_2} in medulla; Q_g did not change in either tissue but L.A. increased somewhat.

When the pH was raised to about 8.1 an additional effect was observed. Increasing the carbon dioxide from 1 per cent to 5 per cent (OP) brought about marked elevation in the Q_{O_2} in cortex. In medulla the elevation was smaller than in cortex and the errors were too large to give the change much significance. Q_g and L.A. increased in both tissues.

IV

The results for aerobic glycolysis in cerebral cortex and medulla oblongata resemble qualitatively those obtained before with sarcoma (anaerobic) and retina (aerobic). That is, the Q_g with 5 per cent carbon dioxide was about double the Q_g with 1 per cent carbon dioxide; the Q_g with 20 per cent carbon

dioxide was the same as with 5 per cent. In absolute terms, smaller effects of carbon dioxide were observed here than with sarcoma and retina. Possibly this is due to the fact that aerobic glycolysis is quantitatively less important in brain than in sarcoma and retina. The effect of carbon dioxide on anaerobic glycolysis in brain and retina remains to be determined.

It is too soon to do more than speculate about the mechanism of the acceleration of glycolysis by carbon dioxide-bicarbonate, but one possibility will be mentioned. Solomon, Vennesland, Klemperer, Buchanan, and Hastings (1941) found in the rat that 1 in 8 carbon atoms in newly formed liver glycogen was derived from bicarbonate carbon. They proposed that this glycogen was synthesized from phosphopyruvic acid formed from malic or fumaric acid (Kalckar, 1939), which arose by way of the Krebs cycle from the combination of carbon dioxide and pyruvic acid (Krebs and Eggleston, 1940, and Evans and Slotin, 1940). An increase in the rate of glycogen formation by these reactions might conceivably promote glycolysis. In brain, glucose rather than glycogen is thought to be the precursor of most of the lactic acid formed; glycogen is broken down to lactic acid to a limited extent, however (Ashford, 1933). The hypothesis of Ashford and Holmes (1926) that glycolysis in the brain may proceed by two pathways was consistent with the observation that superimposed on the variation in glycolysis with oxygen tension was a decrease in rate from the 1st to the 2nd hour independent of oxygen tension.

Although the synthesis of dicarboxylic acids from pyruvate might possibly account for the action of carbon dioxide-bicarbonate it is not clear how it could account for the action on both respiration and glycolysis. In retina the addition of succinate in a 1 per cent carbon dioxide medium raised the Q_{O_2} to the level observed with 5 per cent carbon dioxide medium but did not increase glycolysis, whereas in 5 per cent carbon dioxide medium both Q_{O_2} and Q_g were higher than in the 1 per cent carbon dioxide medium.

The sensitivity of glycolysis to carbon dioxide has certain physiological implications. Inhalation of carbon dioxide in the appropriate concentrations results in anesthesia or in a considerable alteration of pulmonary ventilation and blood pressure. These responses of the intact animal involve functional changes in the brain which might be associated with alterations in metabolism that could be seen *in vitro*. The concentration of carbon dioxide must rise to about 20 per cent in order to produce anesthesia. There was no significant change in Q_{O_2} or Q_g of cortex when carbon dioxide was increased from 5 per cent to 20 per cent either at constant pH (DN) or at constant bicarbonate (AF). These results agree in general with those for ether. This agent supplied to cat cortex in anesthetic concentrations *in vitro* had no effect on Q_{O_2} , but increased L.A. slightly (Craig, 1943). Such observations tend to support the view that the depression of metabolism of cerebral cortex is not an essential feature of anesthesia.

Pulmonary ventilation may be augmented by increases in the carbon dioxide tension up to 9 per cent of an atmosphere and by decreases in oxygen tension below about 13 per cent, in the inspired air, through the mediation of chemoreceptors in the medulla oblongata, the aortic arch, and the carotid sinuses. Respiratory adjustments to anoxia are probably carried out by means of the peripheral chemoreceptors rather than by the center directly. However, centrally driven anoxic hyperpneas have been consistently observed when the anesthesia was not too deep, so that the mechanism of the central response to anoxia is not without significance (Moyer and Beecher, 1942). According to the original theory of Gesell (1925), the central chemoreceptors respond to increased intracellular acidity brought about in the case of hypercapnia by the diffusion of carbon dioxide into the cells of the chemoreceptors, and in the case of anoxia, by the formation of lactic acid within them. The data for the metabolism of medulla do not necessarily apply to the respiratory neurones (Pitts, Magoun, and Ranson, 1939) scattered through that region. If it is assumed that medulla slices are representative of the respiratory center, however, two comments on the intracellular acidity theory may be made.

In the first place the oxygen tension experiments point to an increase in lactic acid output in anoxia but not necessarily to an increase in intracellular acidity. In the second place the experiments in which the concentration of bicarbonate buffer was varied suggest that in hypercapnia lactic acid output is increased as well as in anoxia. This is of interest in the light of Winder's experience.

Winder (1937) studied the effect of monoiodoacetic acid (MIA) on the ability of the isolated perfused carotid chemoreceptors to respond to anoxia (perfusion fluid in equilibrium with 5.5 per cent carbon dioxide and 0.5 per cent oxygen) and hypercapnia (perfusion fluid in equilibrium with 35 per cent carbon dioxide and 62 per cent oxygen). With concentrations of MIA small enough to avoid poisoning the pressoreceptors, he found in eight of fifteen experiments that the responses to both anoxia and hypercapnia were eliminated; in the remaining seven, the response to hypercapnia persisted. Winder attributed these results to a specific inhibition of glycolysis by MIA. Working with brain tissue, Fuhrman and Field (1943) have shown that in appropriate concentration MIA can block most of the anaerobic glycolysis without inhibiting respiration markedly. The elimination of the hypercapnic response by MIA was difficult to account for on the theory that carbon dioxide acts by virtue of its acidity, and Winder was forced to suggest that glycolysis was in some way involved in the response to hypercapnia as well as to anoxia.

If the present experiments have any bearing upon the mechanism of the hypercapnic and anoxic respiratory responses, it is to call attention to the possibility that glycolysis rather than intracellular acidity may be the common factor. Lactic acid production has one advantage over intracellular pH in

that it can be more directly measured. In evaluating the results obtained with medulla slices, however, it cannot be overlooked that an increase in carbon dioxide tension accelerated glycolysis only when the pH was maintained constant, whereas the hyperpnea of hypercapnia is brought about in the face of a decline in blood pH.

The rate of oxygen uptake may also be of importance in these respiratory responses. Zuntz (1897) has shown that pulmonary ventilation declines when the carbon dioxide tension in the inspired air rises much beyond 15 per cent; with 20 per cent carbon dioxide there was a significant decrease in the oxygen uptake of medulla slices (Table VI). In similar fashion, the anoxic hyperpnea passes through a maximum as oxygen tension is progressively decreased; the decline in breathing in spite of increasing lactic acid output may be related to the fall in Q_{O_2} at low oxygen tension.

SUMMARY

Manometric measurements were made of oxygen uptake (Q_{O_2}) and aerobic lactic acid output (Q_G) by slices of cerebral cortex and medulla oblongata of the cat in the presence of mixtures of 1, 5, and 20 volumes per cent of carbon dioxide in oxygen. The concentrations of NaHCO_3 and NaCl in the medium were varied to maintain constant pH and sodium ion concentrations. The calcium ion concentration was 0.0002 M.

At pH 7.5 under these conditions, an increase in carbon dioxide from 1 per cent to 5 per cent doubled the Q_G of both tissues but did not alter Q_{O_2} ; an increase from 5 per cent to 20 per cent carbon dioxide had no further effect on Q_G in either tissue or Q_{O_2} of cortex, but did depress the Q_{O_2} of medulla.

At pH 8.1, an increase in carbon dioxide from 1 per cent to 5 per cent raised the Q_{O_2} and Q_G of cortex by about 60 per cent.

Measurements at low oxygen tension carried out previously in phosphate medium were repeated in bicarbonate medium to obtain data for the combined output of lactic acid and carbon dioxide (Q_A). When the oxygen in the gas phase was decreased from 95 to 3 volumes per cent, the lactic acid output as measured colorimetrically increased by 114 mg./gm. in cortex and by 8 mg./gm. in medulla; Q_A increased from 12.3 to 13.5 in cortex and decreased from 5.1 to 3.8 in medulla.

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I. THE RELATION BETWEEN ATTACHMENT TO THE SUBSTRATUM AND INGESTION BY AMEBA IN STRYCHNINE SULFATE SOLUTION AND CONDITIONED MEDIA*,†

II. BIOLOGICAL ASSAY OF CONDITIONED MEDIA

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PLATE 2

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Under certain conditions, amebae ingest food, are attached to the substratum, and numerous inactive *Chilomonas paramecium* adhere to the plasmalemma; under other conditions, some ingest no food, are unattached, and no chilomonads adhere to the plasmalemma. Mast and Hahnert (1935) contend that ingestion is dependent on attachment, and Mast and Fennell (1938) state (p. 15): "while the effect of non-electrolytes on attachment seems to be the same as it is on ingestion, the effects of salts and hydrogen ions on ingestion differ greatly from their effects on attachment, and that this is particularly striking in reference to a hydrogen ion concentration of pH 7, in which ingestion was found to be maximum and attachment minimum.

This strongly indicates that, while ingestion is doubtless dependent on attachment, in accord with the views of Mast and Hahnert (1935), these two phenomena are not closely correlated".

Aside from these observations nothing has been reported regarding the relation between attachment to the substratum, adherence of inactive chilomonads to the plasmalemma, and ingestion of food by ameba.

Little is known about the effects of strychnine on physiological processes in the Protozoa. Kriz (1924) contends that strychnine sulfate ultimately inactivates *Chilodon megalotrocha* by stopping ciliary activity, and Swindle and Kriz (1924) maintain that in *Vorticella* strychnine first decreases, then stops ciliary activity. Additional information with regard to the effects of strychnine on physiological processes in the Protozoa, especially on attachment and ingestion in the rhizopod Protozoa, is highly desirable.

More is known, however, concerning the effects of conditioned media on animal aggregations, and Allee (1931) extensively reviews and summarizes the literature in this field.

The results obtained in this investigation are presented under three headings:

* Some of the observations were made at the Marine Biological Laboratory, Woods Hole, Massachusetts.

† A part of this investigation was aided by a grant from the Society of Sigma Xi.

- (1) The relation between adhesiveness of the plasmalemma, attachment to the substratum, and ingestion of inactive chilomonads by *Amoeba proteus*. (2) The relation between conditioned media, attachment to the substratum, and ingestion of food by *Pelomyxa carolinensis*. (3) Biological assay of conditioned media.

Material and Methods

C. P. chemicals and water redistilled in pyrex glass were used in the preparation of all inorganic salt solutions. All inorganic salts were carefully dried and weighed. The strychnine sulfate was obtained from the chemical supply room of the Marine Biological Laboratory.

Amoeba proteus and *Pelomyxa carolinensis* were cultured in Chalkley solution.¹ *Chilomonas paramecium* was cultured in sterile glucose-peptone solution, and *Colpidium striatum* was grown in sterile tryptone solution. *Paramecium caudatum* was cultured in hay infusion-lettuce medium. For other details regarding culture methods, media used, preparation of organisms for experimentation, consult Mast and Fennell (1938).

The standard deviations (σ) and the (t) value used for evaluating the results are calculated by the methods of Baten (1938). The results obtained are given in the following pages.

The Relation between Adhesiveness of the Plasmalemma, Attachment to the Substratum, and Ingestion of Chilomonads by Amoeba proteus

In each of the following experiments 20 to 30 amebae were used, and most experiments were repeated from 2 to 5 times. *Chilomonas paramecium* was used as the food organism for studying ingestion by amebae in strychnine sulfate solution. Tables I and II, Text-fig. 1, and Figs. 1 to 3 give a summary of the results obtained.

Table I, column 6, shows that in solutions of single salts to which strychnine sulfate was added until its concentration was 0.000069 M, percentage attachment of amebae to the substratum in 20 minutes is highest in $MgCl_2$ solution (83.3), lower in $CaCl_2$ solution (68.0), and lowest in KCl solution and NaCl solution, in which it is 2.5 and 1.3 respectively. Table I, column 12 demonstrates that when strychnine sulfate is omitted, percentage attachment in 20 minutes is highest in $MgCl_2$ solution (85.0), next highest in NaCl solution (77.3), lower in $CaCl_2$ solution (73.3), and lowest in KCl solution (40.8). The results obtained in the latter group of solutions are in general agreement with those obtained by Mast (1929). Further analysis of the results in columns 6 and 12, makes it apparent that percentage attachment by amebae in $CaCl_2$ or $MgCl_2$ solution containing strychnine is about the same as percentage attachment in the corresponding control solutions.

¹ Chalkley solution: 80 mg. NaCl, 4 mg. $NaHCO_3$, 4 mg. KCl, 4 mg. $CaCl_2$, 2 mg. $CaH_4(PO_4)_2$, 2 mg. $Mg_3(PO_4)_2 \cdot 4H_2O$, and 1,000 cc. distilled water.

TABLE I

The Relation between Strychnine Sulfate in Solutions of Single Salts and Attachment of Amoeba proteus to the Substratum

1	2	3	4	5	6	7	8	9	10	11	12
Composition of experi- mental solution	No. of amebae tested	No. of amebae attached			Per- centage attach- ment in 20 min.	Composition of control solution	No. of amebae tested	No. of amebae attached			Per- centage attach- ment in 20 min.
		Time in min.						Time in min.			
		5	10	20				5	10	20	
NaCl 0.0029 M plus strychnine sulfate 0.000069 M	150	6	3	2	1.3	NaCl 0.0029 M	150	70	101	116	77.3
KCl 0.0029 M plus strychnine sulfate 0.000069 M	120	1	3	3	2.5	KCl 0.0029 M	120	22	60	49	40.8
CaCl ₂ 0.002 M plus strychnine sulfate 0.000069 M	150	93	89	102	68.0	CaCl ₂ 0.002 M	150	92	101	110	73.3
MgCl ₂ 0.002 M plus strychnine sulfate 0.000069 M	60	40	44	50	83.3	MgCl ₂ 0.002 M	60	40	50	51	85.0

TABLE II

The Relation between Strychnine Sulfate in Solutions of Single Salts and Frequency of Ingestion of Food by Amoeba proteus

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Composition of solution tested	Time in min.						Composition of control solution	Time in min.							
	10	20	10	20	10	20		10	20	10	20	10	20	10	20
	No. of amebae tested		Mv.* for chilo- monads in- gested by one ameba		θ	θ		No. of amebae tested		Mv. for chilo- monads in- gested by one ameba		θ	θ	‡	
0.0029 M NaCl plus 0.000069 M strychnine sulfate	44	43	29	46	8.06	15.92	0.0029 M NaCl	46	45	13	26	6.34	8.75	10.45	7.79
0.0029 M KCl plus 0.000069 M strychnine sulfate	50	45	21	45	7.48	12.70	0.0029 M KCl	65	64	9	15	9.02	14.50	7.79	11.45
0.002 M CaCl ₂ plus 0.000069 M strychnine sulfate	21	22	27	38	7.58	11.47	0.002 M CaCl ₂	21	24	11	22	5.91	7.35	7.63	5.59
0.002 M MgCl ₂ plus 0.000069 M strychnine sulfate	56	53	23	31	6.38	8.24	0.002 M MgCl ₂	52	58	19	30	8.45	10.36	2.77	.53

* Mv., arithmetic mean.

† t value, the difference between the means divided by the standard error of that difference. When the t value is over the 2.6 the results are considered significant.

A transposition of the results given in Table II, columns 5 and 12, from total number of chilomonads ingested in 20 minutes to frequency of ingestion per minute, makes it perceivable that ingestion is increased from 1.3 to 2.3 chilomonads per minute when strychnine is added to NaCl solution. In like manner strychnine increased ingestion in KCl and CaCl₂ solutions. Ingestion is increased in the former from 0.75 to 2.25 and in the latter from 1.1 to 1.9 chilomonads per minute. The most striking difference between the foregoing results and those obtained by adding strychnine to MgCl₂, is that frequency in the latter solution, over a period of 20 minutes, is about equal to the frequency in the corresponding control solution. Figs. 1 to 3 offer suggestions as to why such differences in frequency are observed.

It is discernible in Fig. 1, that in NaCl containing strychnine large numbers of chilomonads adhere to the plasmalemma of amebae, to cotton fibers, and in this adherence clumps are formed. In KCl and CaCl₂ solutions, strychnine likewise increased adherence of chilomonads to the plasmalemma of *Amoeba*. Fig. 2 shows that strychnine had little effect on adherence in MgCl₂ solution. These observations suggest that increased ingestion is closely correlated with adherence of chilomonads to the plasmalemma.

A comparison of the results presented in Table II, column 5, with those presented in Table I, column 6, shows that under certain conditions, *i.e.* when strychnine is added to NaCl and KCl solutions, attachment to the substratum and ingestion are not closely correlated. Under other conditions, attachment and ingestion are fairly closely correlated; *i.e.*, in CaCl₂ and MgCl₂ solutions.

The supplemental photomicrograph, Fig. 3, shows an ameba in strychnine sulfate solution. It can be seen that this individual is oval, and that numerous short pseudopods, which are rounded at the distal ends, project from the surface. It is repeatedly observed that amebae responded in this way to strychnine.

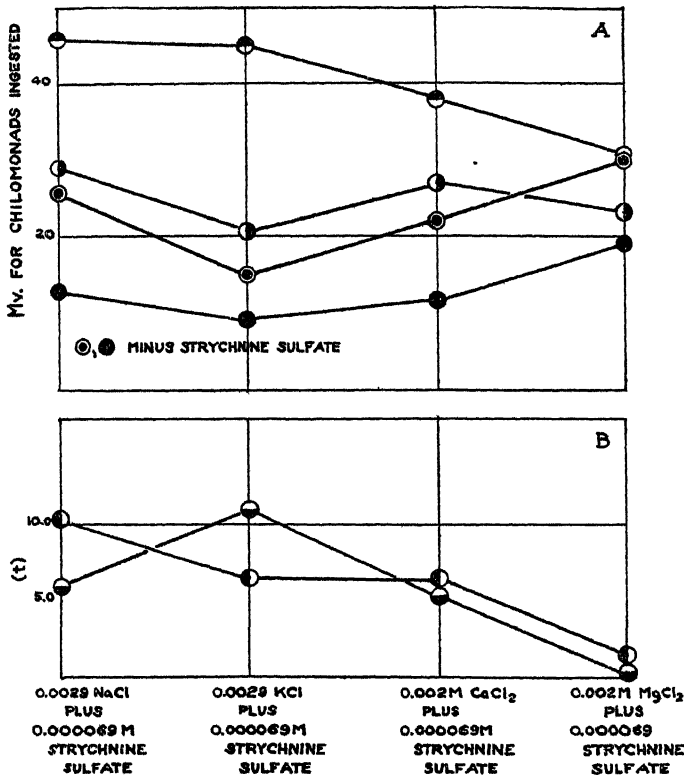
The (*t*) values given in Table II, columns 15 and 16, and Text-fig. 1 show that strychnine sulfate in NaCl, KCl, and CaCl₂ solutions significantly increase frequency of ingestion by *Amoeba proteus*. It is obvious that increased ingestion in MgCl₂ solution containing strychnine is of significance for 10 minutes, but of no significance in 20 minutes.

The Relation between Conditioned Media, Attachment to the Substratum, and Ingestion by Pelomyxa carolinensis

Extract of paramecia (conditioned media) described in the following pages is made by leaving 0.5 cc. of *Paramecium caudatum* in 4 cc. of Chalkley solution for 4 hours, after which all organisms were removed by filtering. Essentially the same procedure was used for preparing extracts from *Chilomonas paramecium* and *Colpidium striatum*.

The method used for preparing alcoholic-ether extracts of paramecia is similar to that used by Roughton (1935) for preparing carbonic anhydrase from red blood cells. To a 15 cc. centrifuge tube containing 0.8 cc. of washed paramecia 2 cc. of distilled

water and 2 cc. of ethyl alcohol are added, after which the tube is shaken vigorously; then 3 cc. of chloroform is added to the tube, and it is again shaken. The tube is then centrifuged at about 1500 R.P.M. for 15 minutes. At the completion of the process a



TEXT-FIG. 1. Effect of strychnine sulfate on frequency of ingestion of food by *Amoeba proteus*. Ordinates in A, arithmetic mean (mv.) for ingestion of food, and in B, ordinates, significance of results (*t*); abscissae, molar concentrations of solutions; ●, mv. in control solution in 10 minutes (salt solution minus strychnine sulfate); ○, mv. in control solution in 20 minutes; ⊙, mv. in test solution in 10 minutes (salt solution plus strychnine sulfate); ⊗, mv. in test solution in 20 minutes; ⊙, significance of results (*t*) in 10 minutes; ⊗, significance of results (*t*) in 20 minutes.

three phase system is established; a clear solution on top, protein in the center, and chloroform at the bottom. By means of a clean pipet, the clear solution is transferred to a small pyrex dish. The dish is put into an oven, and the solution evaporated to dryness at a temperature of 50°; then 3 cc. of Chalkley solution is added to the residue. This solution is described in the following section as alcoholic-ether extract of paramecia. The material designated as good culture solution is Chalkley solution from a

culture in which amebae and other organisms are abundant. All other methods used are similar to those described in the preceding pages. The results obtained are given in Tables III, IV, and V.

TABLE III

Attachment to the Substratum and Ingestion by Pelomyxa carolinensis in Solutions of Dead Organisms and in Extracts of Living Organisms

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Composition of solution tested	No. of amebae tested	No. of amebae attached				Percentage attachment in 60 min.	Ingestion of particles	Composition of control solution	No. of amebae tested	No. of amebae attached				Percentage attachment in 60 min.	Ingestion of particles
		Time in min.								Time in min.					
		10	20	30	60					10	20	30	60		
Chalkley solution plus dead chilomonads	243	74	94	116	132	54.3	+	Chalkley solution	221	4	8	6	5	2.2	x
Chalkley solution plus dead <i>Colpidium</i>	74	26	57	57	63	85.1	+	Chalkley solution	52	1	0	0	0	0	x
Chalkley solution plus dead <i>Paramecium</i>	145	9	45	77	95	65.5	+	Chalkley solution	140	0	0	0	0	0	x
Chalkley solution plus chilomonad extract	230	1	16	30	76	33.0	x	Chalkley solution	218	3	3	2	0	0	x
Chalkley solution plus boiled <i>Paramecium</i> extract	65	0	6	18	56	86.1	x	Chalkley solution	58	0	1	0	0	0	x
Chalkley solution plus <i>Paramecium</i> extract	223	44	97	141	161	72.2	x	Good culture solution	209	3	19	52	90	43.0	x
Chalkley solution plus alcoholic-ether extract of <i>Paramecium</i>	126	0	0	8	78	61.9	x	Chalkley solution	124	0	0	0	0	0	x
Good culture solution plus an equal volume of distilled water	74	0	0	0	4	5.4	x	Good culture solution	159	0	0	6	20	12.6	x
Chalkley solution (10 cc.) plus 10 γ acetylcholine bromide	107	10	14	28	64	59.8	x	Chalkley solution	78	18	19	18	24	30.7	x
Chalkley solution (10 cc.) plus 20 γ of acetylcholine bromide	125	30	31	41	86	68.8	x	Chalkley solution	125	17	18	25	39	31.2	x

+, ingestion of dead organisms; x, ingestion not ascertained.

The results presented in Table III, columns 7 and 15, reveal that percentage attachment to the substratum is increased by adding dead organisms, such as *Paramecium*, *Chilomonas*, or *Colpidium* to Chalkley solution. It is also obvious that chilomonad extract, boiled chilomonad extract, good culture solution, and Chalkley solution containing materials prepared by alcohol-ether extraction

likewise increase percentage attachment to the substratum. It seems rather noteworthy that percentage attachment is decreased from 12.6 to 5.4 when good culture solution is diluted with an equal volume of distilled water. This suggests that attachment is closely correlated with concentration of organic substances present in solutions in which organisms have lived. Other data in columns 7 and 15 support this view. When the concentration of organic substances is increased by adding 2 cc. of *Paramecium* extract to 3 cc. of good culture solution, percentage attachment is increased from 43.0 to 72.2.

TABLE IV

The Relation between Several Types of Inorganic Salts and Attachment of Pelomyxa carolinensis to the Substratum

1	2	3	4	5	6	7	8	9	10	11	12	13	14
Composition of solution tested	No. of amebae tested	No. of amebae attached				Percentage attachment in 60 min.	Composition of control solution	No. of amebae tested	No. of amebae attached				Percentage attachment in 60 min.
		Time in min.							Time in min.				
		10	20	30	60				10	20	30	60	
0.00145 M NaCl	179	10	32	41	57	31.8	Chalkley solution	179	15	38	23	23	12.8
0.0029 M NaCl	224	2	1	2	7	3.1	Chalkley solution	239	0	0	0	0	0
0.00145 M KCl	109	0	0	0	0	0	Chalkley solution	58	0	0	0	0	0
0.0029 M KCl	131	0	0	1	0	0	Chalkley solution	109	3	3	1	0	0
0.001 M CaCl ₂	59	8	7	3	4	6.7	Chalkley solution	48	0	0	0	0	0
0.002 M CaCl ₂	106	5	7	4	0	0	Chalkley solution	78	0	0	0	0	0
0.001 M MgCl ₂	54	0	0	0	0	0	Chalkley solution	50	0	0	0	0	0
0.002 M MgCl ₂	76	1	1	1	4	5.2	Chalkley solution	72	0	1	1	0	0

Further analysis of the results presented in Table III, column 7, shows that in Chalkley solution in which chilomonads were left and then removed, 76 of 230 (33 per cent) amebae are attached in 60 minutes, and that in Chalkley solution containing materials prepared from paramecia by alcohol-ether extraction, 78 of 126 (61.9 per cent) amebae (*Pelomyxa*) are attached in 60 minutes. It is likewise evident that acetylcholine bromide increases percentage attachment to the substratum. The data presented in the preceding section show that protozoan-conditioned media increase both attachment and ingestion in *Pelomyxa*.

The results given in Table IV, columns 7 and 14, show that percentage attachment is higher in 0.00145 M NaCl (31.8), 0.001 M CaCl₂ (6.7), 0.002 M

MgCl₂ (5.2), 0.0029 M NaCl (3.1) than it is in the respective control solutions in which it is 12.8, 0, 0, 0. Increased percentage attachment in 0.00145 M NaCl (31.8) and in the corresponding control solution (12.8) doubtless depends on (1) physiological condition of the amebae used in the experiment and (2) salt effect. Mast and Fennell (1938) who studied ingestion by *Amoeba proteus* in solutions of single salts found that ingestion was higher in NaCl than it was in either KCl, CaCl₂, or MgCl₂ solutions. An analysis of the data given in Table IV will show, however, that inorganic salts, in the concentrations used in the experiments are relatively unimportant factors in facilitating attachment in *Pelomyxa carolinensis*.

Biological Assay of Materials in Solutions in Which Protozoa Have Lived

Tests for Choline Esters.—It has been shown in the preceding pages that acetylcholine bromide increased percentage attachment of *Pelomyxa* to the substratum. Bayer and Wense (1935) contend that they isolated acetylcholine and choline from paramecia.

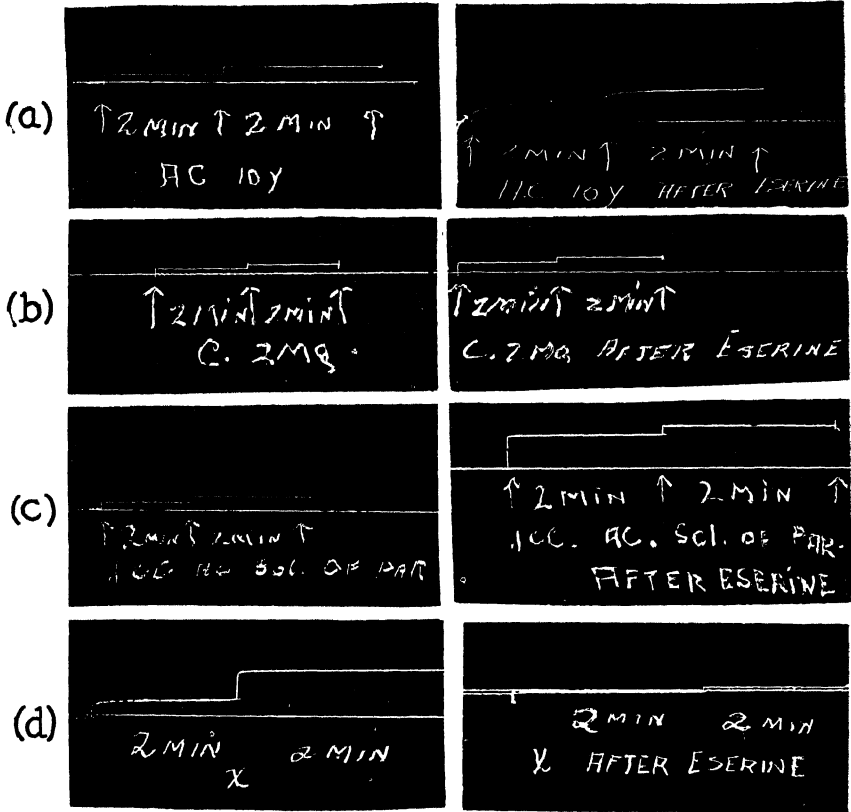
Aqueous solutions of paramecia which were tested for choline and acetylcholine were prepared in the following manner: From 0.1 to 0.25 cc. of washed paramecia were left in 1.5 cc. of acidulated Chalkley solution in a 15 cc. centrifuge tube for 2 hours. Then the centrifuge tube containing the paramecia was transferred to a beaker to which 150 cc. of tap water had been added. The beaker was placed in an oven maintained at 50°, and left until all organisms were killed. The material is referred to in the following pages as "aqueous extracts of paramecia."

Alcoholic extracts of paramecia were prepared by adding 5 cc. of acid alcohol (1.5 cc. of 1 M H₂SO₄ to 100 cc. of ethyl alcohol) to 0.1 to 0.5 cc. of washed paramecia. Such alcoholic extracts were left 12 hours, and then evaporated to dryness in an oven at 50°. The residue obtained was dissolved in Ringer's solution, and then it was tested for choline esters with the rectus abdominis of *Rana pipiens*.

Aqueous extracts were tested for choline esters by the method of Chang and Gadum (1933). The rectus was suspended in a 20 cc. bath in Ringer's solution. The aqueous extract was added to the bath in the following manner: 9 cc. of Ringer's solution was put into the bath, and then aqueous extract in Ringer's solution was introduced into the bath with a pipet. The aqueous extract was left in contact with the muscle for 4 minutes, and then the muscle was removed from the bath and put into 18 cc. of Ringer's solution which contained 0.2 mg. of eserine sulfate and left for 15 minutes. Then the muscle was again suspended in the bath in the aqueous extract in Ringer's solution. Contractions of the muscle were recorded on a smoked drum. Essentially the same method was used for testing alcoholic extracts.

The results obtained are shown in Text-fig. 2. It is evident that aqueous extracts produce a slow contraction in the rectus abdominis and that the height of contraction is increased by eserine sulfate. It can likewise be seen that alcoholic extracts of paramecia contain a pharmacologically active material, and that the activity of this substance is not increased by eserization.

Chang and Gaddum contend that the action of tissue extracts is increased by eserine when acetylcholine is present, and that eserine has little effect on the action of choline.



TEXT-FIG. 2. Tension of frog's rectus abdominis in 18 cc. bath. Concentration of eserine sulfate about 10^{-5} . (a) A. C., acetylcholine bromide. (b) choline. (c) 0.1 cc. aqueous solution of paramecia; 0.1 cc. of dead paramecium. (d) X, materials prepared by alcoholic extraction from 0.5 cc of paramecia. Records show height of contraction. X about 3.

Aqueous extracts were further tested for choline esters by adding 1 cc. of saturated Reinecke salt to 2 cc. of aqueous extract. The precipitate obtained was compared with precipitates obtained by the addition of Reinecke salts to commercial acetylcholine, and commercial choline. It was found that the precipitate of the aqueous extract contained an occasional crystal which resembled those obtained from commercial acetylcholine, although the crystals from the aqueous extract were smaller than those from commercial acetylcho-

line. The bulk of the material obtained by precipitating aqueous extracts resembled neither acetylcholine or choline precipitates. There was a tendency

TABLE V

The Effect of Aqueous Extracts of Paramecia on the Reproductive Structures of a Representative Series of Mice

Mouse No.	Description of animal	Ovary	Vagina	Oviduct	Uterus	Size of testes	Epididymis	Seminal vesicle	Interstitial cells
1	Experimental	Follicles	Open	Normal	Normal				
2	Experimental	Follicles	Open	Normal	Normal				
5	Experimental	Follicles	Open	Normal	Normal				
12	Experimental	Follicles	Open	Normal	Normal				
A	Control	Corpora lutea Follicles	Open	Normal	Normal				
B	Control	Corpora lutea Follicles	Open	Normal	Normal				
C	Control	Corpora lutea Follicles	Open	Normal	Normal				
16	Control	Corpora lutea Follicles	Open	Normal	Normal				
4	Experimental					5 × 3 mm.	Cells and detritus	3*	Abundant
11	Experimental					7 × 4 mm.	Cells and detritus	3	Abundant
14	Control					9 × 4 mm.	Cells and detritus	4	Abundant
17	Control					7 × 4 mm.	Cells and detritus	4	Abundant

* Smaller than control and contained less fluid.

for this material to form chains and it glistened more under reflected light than either of the other precipitates.

Some aqueous extracts had little effect on the rectus abdominis. It is likely that the absence of pharmacologically active materials in aqueous extracts may be due to a ferment which destroys such materials, as Wense (1938) contends

that acetylcholine is destroyed in aqueous extracts by an esterase. Chang and Gaddum, who studied tissue extracts, found that acetylcholine is most stable in weak acids. It was also observed that the rectus abdominis in some frogs was more sensitive to the action of extracts than it was in others.

The above results demonstrate that aqueous and alcoholic extracts of paramecia contain under certain conditions pharmacologically active materials which have certain characteristics in common with choline esters.

Tests for Estrogenic Materials.—Steidle (1930) and Bauer (1932) found that alcoholic extracts of Protozoa (the former used *Paramecium* and the latter *Colpoda Steimi*) would produce estrus in castrated female mice. In the experiments to be described two litters of mice (17 animals, 21 days of age) were divided into four groups. Two of the four groups were subcutaneously injected daily with 0.03 to 0.04 cc. of dead paramecia in Chalkley solution for 21 days. Then all mice were autopsied, and the reproductive structures of both sexes fixed in Helly's fluid, and stained with hemotoxylin-eosin. The results obtained are presented in Table V.

Table V and Figs. 4 and 5 show that dead paramecia when injected subcutaneously into mice inhibit development of corpora lutea. Other structures in the female reproductive system appear to be normal. It is evident that the vaginae in all animals were open, and that all ovaries contain follicles. These results indicate that dead paramecia inhibit the action of the luteinizing hormone (LH) of the pituitary. Fevold, Hisaw, and Leonard (1931) showed that pituitary gonadotropic extract contained a follicle stimulating (FSH) and luteinizing (LH) hormones. Several years later (1934) these investigators isolated these two substances, demonstrating that follicle stimulation and luteinization are apparently dependent on separate chemical substances.

Before autopsying the experimental and control animals, blood was removed from the heart of each of them for antibody tests while the animals were under ether anesthesia. The sera of both experimental and control animals were then used for ascertaining antibody content by introducing paramecia into various dilutions of sera. Paramecia when introduced into undiluted antisera were lysed in less than 5 minutes. Antisera when diluted seven times with Chalkley solution lysed paramecia in less than 20 minutes. One part of antisera to ninety-nine parts of Chalkley solution dissolved about 90 per cent of the paramecia added to the solution in 1200 minutes. Paramecia remained active in normal and diluted normal sera. These results when compared with those presented in Table V suggest that production of antibodies by mice in some way inhibits or destroys the luteinizing hormone of the pituitary. Gordon (1937) in his work with hormones does not eliminate the possibility that the effects of antihormones may depend on the antibodies produced by the materials injected.

Aqueous solutions of paramecia were also tested for estrogenic materials by

subjecting castrated female mice to daily injections of 0.15 cc. of dead paramecia in Chalkley solution for 5 days. Vaginal smears were made from these mice, and the smears showed an abundance of epithelial cells and leucocytes. The above observations show that if estrogenic substances are present in solutions of paramecia, the concentration is inadequate for the production of estrus in castrated female mice.

Table V also shows that the most pronounced difference in experimental and control males is the difference in size of the seminal vesicles. Seminal vesicles from the control animals were large and extended with fluid, while the vesicles of experimental animals were smaller and contained less fluid. The epididymis of both experimental and control animals showed masses of spermatozoa and detritus in about the same concentrations. Interstitial cells in both experimental and control animals were abundant.

The observations given in the preceding paragraphs show that non-specific substances may destroy the action of the luteinizing pituitary hormone. The nature of the inhibition is not understood although the observations suggest that non-specific materials produce antibodies which inhibit or destroy the luteinizing hormone of the pituitary.

DISCUSSION

The results presented in the preceding pages show that adhesiveness of the plasmalemma and attachment to the substratum by *Amoeba proteus* are not closely correlated in NaCl and KCl solutions containing strychnine sulfate, and that adhesiveness of the plasmalemma and ingestion are fairly closely correlated. These observations suggest that strychnine sulfate in the concentrations used is toxic to amebae, and this toxicity is sufficient to decrease percentage attachment, yet it is insufficiently toxic to inhibit adherence. Thus, increased ingestion in strychnine sulfate solutions appears to be due to the availability of organisms adhering to the plasmalemma.

Under normal cultural conditions a correlation presumably exists between adhesiveness of the plasmalemma and ingestion of food by *Amoeba proteus*, as it is frequently observed that chilomonads collect on the plasmalemma in cultures in which ingestion is maintained at a fairly constant rate over a period of days. It is also observed that chilomonads are not attracted to the plasmalemma from a distance. Contact is made, when by random movements individuals strike the plasmalemma, and they then adhere to it as though the plasmalemma was covered by a sticky material. Amebae with plasmalemmas in this condition frequently have large numbers of food vacuoles of different diameters, in various stages of digestion in the plasmasol. An interesting hypothesis is that adhesiveness develops when metabolites are excreted to form a thin film of adhesive material on the surface of the plasmalemma. Another factor which may be of importance in increasing adhesiveness is bacterial

products which injure the cell surface. It is probable that increased adhesiveness of the plasmalemma in strychnine sulfate is a result of injury to the cell surface.

A review of the results previously given will show that Chalkley solution or solutions of single salts are relatively unimportant factors in attachment of *Pelomyxa carolinensis* to the substratum. Then, on what does attachment in this species of ameba depend? The following hypothesis is suggested: In cultures in which attachment, ingestion, and fission are accelerated, an optimum concentration of organic material is present in the culture solution. If the organic material increases beyond the optimum, as is the case when ciliates and flagellates reach a high concentration, attachment and ingestion are inhibited. Likewise, if organic materials are decreased to a suboptimum concentration, *i.e.* by diluting a good culture solution (a solution in which *Pelomyxa* are abundant) with distilled water, attachment and ingestion are likewise inhibited.

Wense (1938), who studied the relation between concentration of acetylcholine and temperature in extracts of paramecia, suggests that unfiltered extracts contained esterase inhibitor bodies which inhibited the action of an acetylcholine destroying esterase. This material was found to be in greater concentration in extracts that were left in an incubator than in extracts left in the refrigerator. He interprets these results as an indication of a close correlation between concentration of esterase inhibitor and increased bacterial activity in the incubated extract. Filtered extracts were found to contain fewer bacteria and less inhibitor; and as a consequence, the acetylcholine was destroyed by esterase. If these results correctly describe the relation between organisms and organic materials in culture solutions it is evident that this relationship is complex and highly variable. Assuming that an optimum concentration of acetylcholine is necessary for maximum growth, ingestion, and attachment in *Pelomyxa*, it can be seen that the maintenance of an optimum concentration of acetylcholine would depend on a variety of factors. Under these circumstances, it would be possible to have maximum attachment and ingestion under some conditions, and under other conditions attachment and ingestion would be at a minimum.

SUMMARY

1. Strychnine sulfate 0.000069 M decreased percentage attachment to the substratum by *Amoeba proteus* in 0.0029 M NaCl from 77.3 to 1.3, in 0.0029 M KCl from 40.8 to 2.5, in 0.002 M CaCl_2 from 73.3 to 68.0, in 0.002 M MgCl_2 from 85.5 to 83.3.

2. Frequency of ingestion of chilomonads by *Amoeba proteus* is increased by adding strychnine sulfate to solutions of NaCl, KCl, or CaCl_2 . Frequency of ingestion is increased in NaCl solution from 1.3 to 2.3, in KCl from 0.75 to 2.25, and in CaCl_2 from 1.1 to 1.9 chilomonads per minute. Ingestion is not significantly increased by the addition of strychnine to MgCl_2 solution.

3. Frequency of ingestion of food by *Amoeba proteus* is not closely correlated with attachment to the substratum in NaCl and KCl solutions to which strychnine sulfate is added.

4. Chilomonads adhere to the plasmalemma of *Amoeba proteus* in solutions of NaCl, KCl, or CaCl₂ containing strychnine, but in MgCl₂ plus strychnine only a few adhere to it. Strychnine appears to make the surface of the amebae and chilomonads sticky in the former but not in the latter. Frequency of ingestion is apparently correlated with adherence of chilomonads to the plasmalemma.

5. Attachment to the substratum and ingestion by *Pelomyxa carolinensis* is increased by dead *Chilomonas*, *Colpidium*, and *Paramecium* in aqueous solutions, by materials obtained from paramecia by alcoholic-ether extraction, and by solutions in which these organisms have lived.

6. Attachment to the substratum by *Pelomyxa carolinensis* is not closely correlated with kind or concentration of inorganic salts used in this study.

7. Materials were found in extracts of paramecia which had certain characteristics in common with choline esters. There is no reason to doubt that under certain conditions materials are present in aqueous and alcoholic extracts which are pharmacologically similar to choline and acetylcholine.

8. Aqueous suspensions of paramecia when subcutaneously injected into young mice for 21 days inhibit the gonadotropic luteinizing hormone of the pituitary. Ovaries from injected mice showed no corpora lutea, and the seminal vesicles from injected males were smaller and contained less fluid than those of the controls.

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EXPLANATION OF PLATE 2

Photomicrographs showing the relation between strychnine sulfate in solutions of single salts and adherence of *Chilomonas paramecium* to the plasmalemma of *Amoeba proteus*.

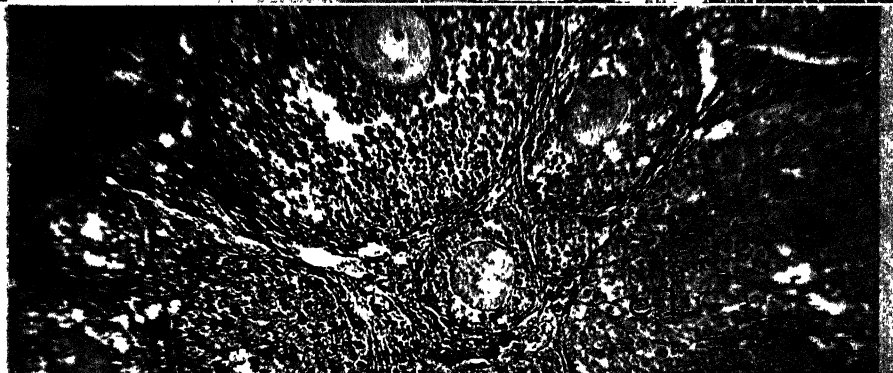
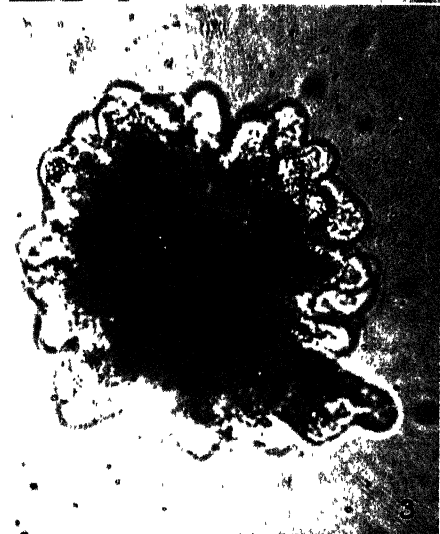
FIG. 1. Ameba in 0.0029 M NaCl plus 0.000069 M strychnine sulfate. $\times 80$.

FIG. 2. Amebae in 0.002 M $MgCl_2$ plus 0.000069 M strychnine sulfate. $\times 50$.

FIG. 3. An ameba in 0.0029 M NaCl (minus chilomonads) plus 0.000069 M strychnine sulfate. $\times 260$.

FIG. 4. Ovary showing follicles, from mouse injected daily for 21 days with aqueous solutions of paramecia. $\times 200$.

FIG. 5. Ovary from mouse C (control) showing follicles and corpora lutea (c.l.). $\times 200$.



STUDIES ON THE ANOMALOUS VISCOSITY AND FLOW-BIREFRINGENCE OF PROTEIN SOLUTIONS

III. CHANGES IN THESE PROPERTIES OF MYOSIN SOLUTIONS IN RELATION TO ADENOSINETRIPHOSPHATE AND MUSCULAR CONTRACTION

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INTRODUCTION

This paper contains the results of an intensive study of the particle shape of the globulin of muscle, myosin, by the methods of anomalous viscosity and flow-birefringence.¹ Our knowledge of the nature of muscular motion has hitherto been divided into two main fields of successful analysis. First, there was the discovery of the phosphorylation cycles whereby energy is transferred from carbohydrate breakdown to the muscle fibre; here the earliest landmarks were the classical investigations of Fletcher and Hopkins (1907) and of Harden and Young (1910)—the papers of Parnas (1937) and of D. M. Needham (1938) may be consulted for up-to-date reviews. Secondly, there was the discovery of the elongated or anisometric character of the particles of myosin, suggested by Brücke (1858) and demonstrated in the classical paper of von Muralto and Edsall (1930). This threw much light on the long known birefringence of intact muscle and its puzzling histology, and it was followed by the application of x-ray techniques to the problem, and the suggestion that muscular contractility is essentially a molecular contractility of protein chains (Astbury, 1933, 1939; Astbury and Bell, 1938; Astbury and Dickinson, 1940; Meyer and Picken, 1937) as Engelmann (1906) had surmised. The exact connection between these two great groups of observations, however, still remained obscure.

In order to bridge this gap, the most promising point of departure seemed to be the important finding of Engelhardt and Ljubimova (1939) that the enzyme adenosinetriphosphatase is either myosin itself or some protein very closely associated with it. This was confirmed by one of us in the following year (see

¹ The present work was begun in 1940 by Joseph Needham, Shih-Chang Shen (Fellow of The Rockefeller Foundation), and Dorothy M. Needham, with A. S. C. Lawrence as rheological adviser. Arnöst Kleinzeller and Margaret Miall joined the group with Rockefeller Foundation grants in 1941, and Mary Dainty (Research Scholar of the Education Authority of the West Riding of Yorkshire) in 1942. Two preliminary reports have already appeared (Needham, Shen, Needham, and Lawrence, 1941; Needham, Kleinzeller, Miall, Dainty, Needham, and Lawrence, 1942).

D. M. Needham, 1942), and shortly afterwards by Szent-Györgyi and Banga, 1941; Edsall and Singher, 1941; Bailey, 1942). Its great importance lies in the following facts.

(1) Among the processes of intermediary metabolism in the muscle, that nearest in time to the contraction of the fibrils is the breakdown of adenosinetriphosphate² to adenosinediphosphate and inorganic phosphate.

(2) This is the only known reaction capable of supplying the bulk of the free energy for contraction. Although some ATP is broken down by transfer of phosphate to hexosemonophosphate (Neuberg ester) (by an enzyme not present in the myosin fraction of the proteins) this also probably liberating free energy, the quantitatively largest part of its breakdown occurs by splitting off of free phosphate under the influence of the enzyme adenosinetriphosphatase (D. M. Needham, 1942). It was considered of interest, therefore, to give careful study to the effect of ATP on the flow-birefringence and anomalous viscosity of myosin. This opinion was strengthened by the fact that after the work had begun, and as it proceeded, other investigators found it impossible, in spite of serious efforts, to separate the adenosinetriphosphatase activity from the protein myosin (Bailey, 1942, confirming the original work of Engelhardt and Ljubimova, 1939).

Since the data in the literature on the physicochemical properties of myosin, though numerous, are rather disjointed, we had to devote a good deal of time to the systematic examination of certain effects, as background for the interpretation of the action of ATP on myosin.

EXPERIMENTAL METHODS

Preparation of Myosin.—Myosin was prepared from rabbit muscle as follows (*cf.* Bailey, 1942). Fresh, ice-cooled muscle was finely minced and the brei extracted at 0° for 1 hour with 5 volumes 0.5 M LiCl containing 0.03 M NaHCO₃. Solid NaHCO₃ was added at intervals during the extraction in order to keep the pH alkaline to bromthymol blue and the mixture was mechanically stirred. After the extraction was finished the mixture was quickly centrifuged and the extractant cleared from suspended particles by filtration through filter paper pulp. The clear liquid was poured into 20 volumes ice cold distilled water; the myosin was precipitated by adjusting the pH of the whole to 6.8–7.0 (measured electrometrically), and leaving it to stand in the ice chest overnight. The precipitated myosin was then collected by decantation and centrifuging and dissolved either by addition of solid electrolyte to bring the final concentration to 0.5 M, or by adding an equal volume of 1 M solution of the electrolyte. Unless otherwise stated once precipitated myosin was used in the experiments. For some experiments the myosin was further purified, precipitating it twice by pouring the clear, filtered solution in 0.5 M NaCl into 20 volumes ice cold distilled water (the

² Hereinafter abbreviated as ATP; adenosinediphosphate as ADP; adenosinemonophosphate (adenylic acid) as AA.

pH of which was carefully adjusted to 6.8–7.0) and leaving the myosin standing for a further 24 hours in the ice chest to complete the precipitation. The protein content of the final solution of myosin in 0.5 M electrolyte was determined by estimating the N content (Kjeldahl) and multiplying the value obtained by 6.03 (Bailey, 1939). Myosin prepared in this way is translucent, with a honey-like appearance, readily splits off phosphate from added ATP, and is highly flow-birefringent. If precipitated at a lower pH (6.4–6.6), the myosin is whitish and pasty; at more alkaline pH (7.6 and higher) the myosin falls out in the form of a transparent gel, which in our experience was enzymically inactive although Bailey (private communication) has found such gels to be enzymically active over several weeks. It was found that the flow-birefringence of myosin prepared in the above way does not depend on the nutrition of the animal. Previous to the standardisation of the above method, myosin was prepared without the continuous checking of the pH; such preparations were occasionally non-birefringent (*cf.* Needham, Shen, Needham, and Lawrence, 1941) and the ratio $\Delta/\text{protein}^8$ varied widely, but with the method described we never failed to obtain flow-birefringence. Some of the experiments quoted below were carried out with myosin preparations which had been precipitated slightly on the acid side.

Preparation of Adenosinetriphosphate.—Adenosinetriphosphate was prepared from rabbit or horse muscle by a modification of the method of Lohmann (1931) as described by D. M. Needham (1942). In some cases the ATP was further purified as described by Kerr and Seraidarian (1941).

Preparation of Adenosinediphosphate.—ADP was prepared by the action of purified myosin on ATP as described by Ljubimova and Pevsner (1941) and Bailey (1942).

Preparation of Inosinetriphosphate.—Inosinetriphosphate was prepared from ATP by a modification of Lohmann's method (1932) as described by one of us (Kleinzeiler, 1942).

Preparation of Inosinic Acid.—Inosinic acid was prepared from horse muscle as described by Ostern (1932).

Preparation of Sodium Triphosphate.— $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 6\text{H}_2\text{O}$ was prepared as described by Huber (1937). Analysis: total P, found 19.95 per cent, calculated 19.55 per cent; 37.2 mg. titrated with 0.1 N HCl (methyl orange): found 1.557 ml., calculated 1.56 ml.; traces of inorganic P in 23.8 mg. Na triphosphate; no precipitate with 0.01 M CoCl_2 (see Neuberg and Fischer, 1937).

Estimation of P.—Inorganic P determinations were carried out according to Fiske and SubbaRow (1925).

FLOW-BIREFRINGENCE AND ANOMALOUS VISCOSITY

The apparatus used was exactly as described in a previous paper of this series (Lawrence, Needham, and Shen, 1944), consisting of a small annular cell with rotating external cylinder, mounted on the stage of a polarising microscope; and a coaxial viscosimeter with glass bottom permitting optical measurements of flow-birefringence. Four variables are observed: (a) the intensity

⁸ See p. 360.

of flow-birefringence itself; (b) the angle of isocline; (c) the extent of the anomalous viscosity, that is, the variation of apparent viscosity with rate of shear; (d) the relative viscosity, η/η_0 , where η is the lower limiting viscosity independent of rate of shear at higher rates of shear. The flow-birefringence and the anomalous viscosity of myosin solutions, are, of course, taken to indicate that the particles are of considerable asymmetry; *i.e.*, very much longer than they are broad.

The intensity of flow-birefringence in a protein sol subjected to shear stress depends upon the number of anisometric particles present (protein concentration), the degree of optical anisotropy of the particles themselves, the perfection of their orientation (a resultant of the opposing forces of shearing stress and thermal disorientation), the depth of solution through which the light beam passes, and the other conditions governing particle shape and size such as pH and salt concentration. We express it here in terms of Δ° ; the angle through which the analysing Nicol must be rotated to extinguish the plane-polarised light emerging from a quarter-wave plate. The double refraction itself can be obtained at once from this figure by means of the following relations:

$\frac{\Delta^\circ}{180^\circ} = \Delta\rho$ the phase difference in wave-lengths between the two components of the elliptically polarised light; and $\frac{\Delta\rho\lambda}{S} = n_e - n_o$ the double refraction, where λ is the wave-length of the light source, and S the depth of solution under examination.

The angle of isocline ψ , is by definition the larger of the two angles which the cross of isocline makes with the crossed planes of polarisation of the polarising and analysing Nicols. It is here interpreted as a measure of the degree of perfection of the orientation of the anisometric particles in the stream lines under shear stress. It varies between 45° for nil orientation, and 90° for perfect orientation.

The relative viscosity η/η_0 is the ratio of the viscosity of the sol to that of distilled water or equivalent electrolyte solution, at the same temperature. Anomalous viscosity is the departure of viscosity from independence of the shear rate. Unfortunately no satisfactory means has yet been devised for its quantitative expression.

GENERAL INTERPRETATIONS

In order to avoid constant repetition in discussing the interpretation of the effects described in what follows, it is desirable to summarise the various logical possibilities at this point in the form of a table (Table I). The table assumes that flow birefringence, angle of isocline $> 45^\circ$, and anomalous viscosity, are present together, as they are in the case of myosin, and in most proteins which show (in bulk phase) any one of these properties. Anomalous viscosity has,

TABLE I

Possible Variations in Flow-Birefringence and Viscosity Properties of Protein Sols

Δ^0, ψ^0 , flow-birefring- ence; angle of isocline and anomalous viscosity	η/η_0 , relative viscosity	Interpretation
A Rise		Lengthening of axial ratio of rod-like or fibrillar particles by (1) linear aggregation of spherical particles or (2) sliding extension of parallel-aggregated micelles or (3) extension of aperiodic coil, such as denatured polypep- tide chain or (4) true relaxation or extension of molecule or micelle ana- logous to β -, α -, and supercontracted keratin trans- formations
B Fall		(a) Shortening of axial ratio of rod-like or fibrillar particles, by reversal of any one of the processes (1), (2), (3), (4) above, or some other, or (b) Coalescence of rod-like or fibrillar particles into roughly symmetrical tangles of much larger size. This is prob- ably not a reversible process
C	Rise	Either increase of intermicellar forces or, less probably, hydration, with increasing diameter of water shell
D) Fall	Fall	Converse of (C)
E) Rise	Rise	In practice, case (A) always takes this form, since relative viscosity is, in some way as yet not clear, proportional to axial ratio (e.g. ATP recovery; high temperatures)
F Fall	Fall	Converse of (E). Owing to intermicellar forces working in a contrary direction, however, rise or fall of relative vis- cosity may not be what would be expected from the flow- birefringence change which it accompanies (e.g. mono- valent cations at low molarity; divalent cations at high molarity; $\text{PH} < 6$; ATP; urea at high molarity)
G) Rise	Fall	Not so far observed
H) Fall	Rise	Disaggregation of, or formation of tangles from, rod-like or fibrillar particles, accompanied by increase in inter- micellar forces or hydration (e.g. monovalent cations at high molarity; divalent cations at low molarity; pH > 10)
I Constant	Rise or fall	Changes in intermicellar forces, unaccompanied by changes in the axial ratio of the particles; not so far observed
J) Rise	Constant	Not so far observed
K) Fall	Constant	Decrease of axial ratio of the particles, accompanied by compensatory increase in intermicellar forces. (pH 8.5-10; urea at low molarity)

however, been described without flow-birefringence, in the case of a liver nucleoprotein, by Greenstein and Jenrette (1940). Conversely, flow-birefrin-

gence has been described without anomalous viscosity in casein sols at alkaline pH (Nitschmann, 1938; Nitschmann and Guggisberg, 1941). These at present very exceptional cases have been discussed in the first paper of this series.

As will be seen below, most of the possible types of behaviour have been met with in the study of myosin sols. The importance of intermicellar forces is readily appreciated when one remembers the well known fact that during proteolysis, very minute changes in the chemical structure of a protein are accompanied by large changes in the solution's viscosity (Northrop, 1929; Cannan and Muntwyler, 1930). For example, in the peptic digestion of gelatin, the relative viscosity falls to half its original value in $1\frac{1}{2}$ hours but the number of amino groups set free per molecule does not attain its maximum till 356

TABLE II

Relation of Flow-Birefringence to Protein Concentration in Myosin Sols (Δ° /Protein Ratio)

Preparation No.	No. of precipitations	Protein content	Δ at 500 R.P.M.	Δ /protein ratio
		<i>per cent</i>		
1	1	3.02	81°	26.8
	3	2.29	63°	27.5
2	1	2.97	67°	22.6
	3	3.22	92°	28.6
3	1	1.90	49°	26.0
	3	2.02	54°	26.7
4	3	2.23	47°	21.1
5	1	1.79	55°	30.7
	3	0.81	27°	33.2
6	1	1.54	31°	20.4
	3	1.67	39°	23.4
7	1	1.49	35°	23.5
8	1	1.21	34-36°	29.0
9	1	2.44	70°	28.7
10	1	1.62	42°	26.0

hours and its half-value till about 40 hours. This subject has been interestingly discussed by Robertson (1928), Pauli and Valkó (1933), and recently by Mark (1941).

PHYSICOCHEMICAL PROPERTIES OF MYOSIN

The Relation of Flow-Birefringence to Protein Content

It was found that the Δ /protein ratio is of the same order in different preparations of rabbit myosin (see Table II) and does not change appreciably by several reprecipitations. The flow-birefringence was measured in the microscope cell at 500 R.P.M.

The flow-birefringence of anisometric particles is a function of the particle length. It can therefore be concluded that in myosin solutions prepared in the

above way the particle length is of uniform order, and the Δ /protein ratio should be considered one of the physicochemical constants of the protein.

The flow-birefringence of myosin solutions increases in a linear manner with the protein concentration (see Fig. 1; Experiment app/347). This confirmed the results of von Muralt and Edsall (1930).

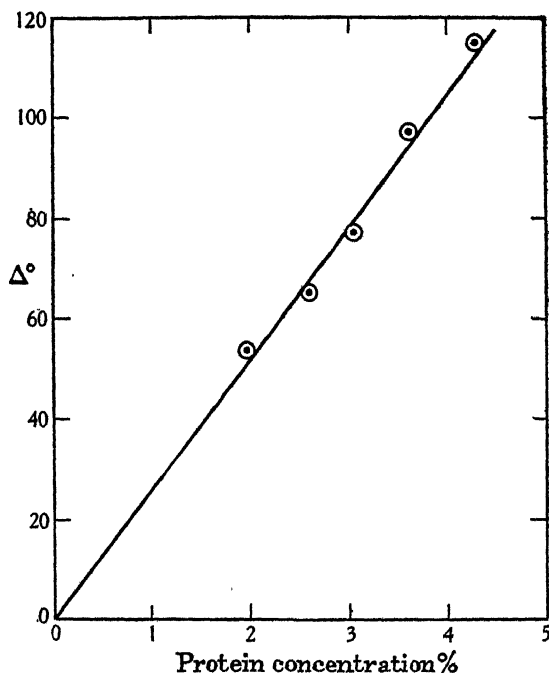


FIG. 1. Relation of flow-birefringence of myosin to protein concentration.

The Relation of Viscosity to Protein Content

As is known, myosin is a protein of extremely high viscosity. From many of our experiments, the relative viscosity can be plotted against the protein concentration (see Fig. 2); it can be seen that it is impracticable to work with myosin solutions of higher concentration than 1 per cent. The viscous anomaly also increases with rising protein concentration (Experiment II 127). Compared with the relative viscosities of other proteins, as in the inset plot in Fig. 2, (taken from the data in the literature, as summarised by Pauli and Valkó, 1933, p. 240), the order of decreasing viscosity is seen to be myosin > sodium caseinate > serum euglobulin > serum pseudoglobulin > serum albumin > ovalbumin; an order which roughly corresponds with the degree of

anisometry of their molecules. That the viscosity does correspond in this way we know from the work of Eirich, Margaretha, and Bunzl (1936) who obtained a graph similar to Fig. 2 using model particles of known lengths.

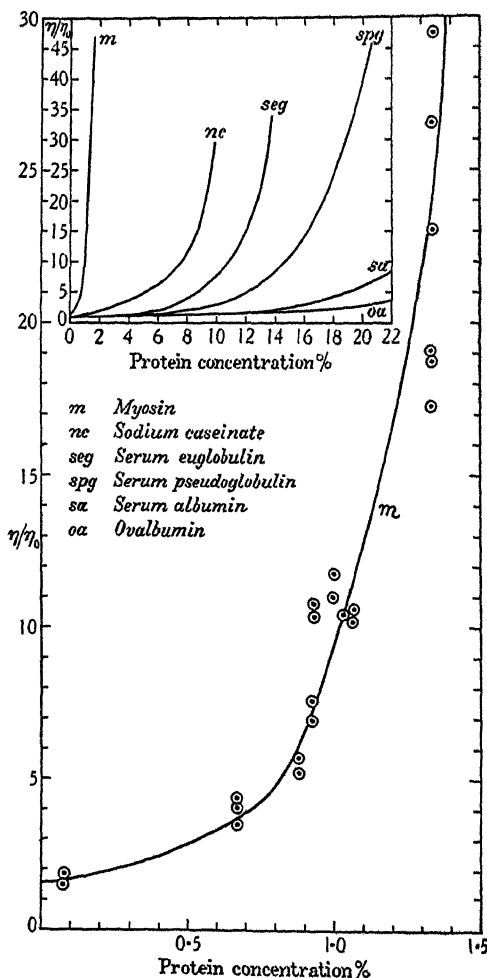


FIG. 2. Relation of relative viscosity of myosin and other proteins to protein concentration.

Effect of Electrolytes on the Flow-Birefringence and Viscosity of Myosin Solutions

The effect of electrolytes on the flow-birefringence of myosin was studied by Edsall and Mehl (1940). In our experiments a more systematic study of the effect of varying concentrations of different electrolytes on the (simultaneously measured) Δ and η/η_0 of myosin solutions was made. The effect of the electrolytes on the Δ was carried out either in the microscope cell, or, together with

measurements of η , in the Couette viscosimeter. The effect of electrolytes was studied either by comparing the Δ and η/η_0 in a number of myosin solutions containing different amounts of electrolyte, but the same protein content; or, where only small amounts of electrolytes were added, by addition of increasing amounts of the salts (in concentrated solutions) to the myosin solution in the viscosimeter, and correcting the values obtained for the dilution of the myosin solutions with the added electrolytes. It should be noted that some of the reported effects of the electrolytes may be due to changes of the pH in the little buffered protein solution.

Effect of Cations

Effect of 0.5 M Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺, NH₄⁺.—The effect of Li⁺ and Na⁺ added to myosin solutions in 0.5 M KCl had been studied by Edsall and Mehl (1940), who found that both electrolytes had decreased the flow-birefringence of

TABLE III

Effect of Cations on the Flow-Birefringence and Relative Viscosity of Myosin (Experiment III 191)

Final concentration of myosin solution 1.86 per cent protein.

0.5 M electrolyte (final concentration)	Δ at 50 R.P.M.	η/η_0 at 2 R.P.M.
LiCl.....	54°	10.5
NaCl.....	53°	8.2
KCl.....	54°	10.5
RbCl.....	55°	10.5
CsCl.....	56°	12.4
NH ₄ Cl.....	42°	—

different myosin preparations. In our experiments the effect of 0.5 M LiCl, NaCl, KCl, RbCl, CsCl, and NH₄Cl on the Δ and η was examined on the same myosin solution. Equal amounts of once precipitated myosin were dissolved in equimolecular quantities of the above salts. The protein content of the solution was 1.86 per cent. The flow-birefringence was measured in the coaxial viscosimeter at 50 R.P.M.; the relative viscosity was calculated (from the values of viscosity obtained at different speeds) for 2 R.P.M. As shown in Table III, the 0.5 M electrolytes have no appreciable effect on the flow-birefringence, and the relative viscosity is affected to a slight degree only. The lowest values of η/η_0 were found for myosin dissolved in 0.5 M NaCl, the highest in 0.5 M CsCl.

Effect of Increasing Concentrations of Li⁺ and K⁺

The effects of increasing concentrations of LiCl and KCl on the Δ and η/η_0 of myosin solutions were studied. To equal volumes of stock myosin solution or myosin gel varying amounts of solid electrolyte were added and the volumes were adjusted by addition of distilled water.

Increasing concentrations of both LiCl and KCl decrease the flow-birefringence of myosin solutions. The highest values of Δ were always observed at about 0.5 M electrolyte; with increasing concentrations the Δ generally falls steadily and reaches 0° at approximately 3.5 to 4.0 M, when the myosin is nearly completely salted out. In a number of experiments a slight increase of flow-birefringence was observed, reaching a maximum somewhere between 0.5 to 1.5 M and then continuing its fall. No explanation can as yet be offered for this observation, which needs further confirmation on more purified myosin. The relative viscosity also decreases as the salt concentration rises, but only to 1.5 to 2.0 M; with higher concentrations a considerable increase of η/η_0 occurs. Here the viscous anomaly, however, is completely lost. The data

TABLE IV

Effect of Increasing Concentrations of LiCl on the Δ and η/η_0 of Myosin Solutions. (Experiment III 181).

Final Concentration of Myosin Solution 1.08 per cent Protein

LiCl concentration M	Δ at 50 R.P.M.	η/η_0 (2.5 R.P.M.)	Viscous anomaly
0.5	67°	9.0	+
0.75	66°	8.2	+
1.0 $\frac{1}{2}$	55°	7.9	+
1.25	20°	7.2	\pm
1.5	8°	6.8	—
2.0	10°	6.2	—
3.0	9°	8.9	—
4.0	0°	10.2	—

in Table IV represent a typical experiment where no transient increase of Δ between the concentrations 1.0 to 1.5 M was observed. Very similar pictures are obtained with potassium chloride. It is interesting that very concentrated salt solutions will decrease the birefringence of the intact muscle fibre also (Nasse, 1882; von Ebner, 1882).

The changes here described for myosin are not, it seems, paralleled very closely by TMD virus under similar conditions. According to Lauffer (1938 *b*) the higher the ionic strength of the electrolyte present, the lower falls the η/η_0 of the virus solution. There is no rise at the higher concentrations of salt, as here.

Effect of NH_4^+

It has long been known that in the presence of ammonia the birefringence of the intact muscle fibre decreases and disappears (Schipilov and Danilevsky, 1881; Biedermann, 1927; Liang, 1936). It has also been shown by Edsall and

Mehl (1940) that 1.4 M NH_4Cl or 1.6 M $(\text{NH}_4)_2\text{SO}_4$ rapidly decreases the flow-birefringence of myosin solutions. In our experiments the effect of increasing concentrations of NH_4Cl on the Δ of myosin solutions was studied in the microscope cell. As shown in Table V, 0.1 M NH_4Cl has no appreciable effect on Δ ; 0.5 M reduces it to 80 to 63 per cent of the control (myosin in 0.5 M LiCl), (see also Table III) whilst no birefringence was observed at 2.0 M NH_4Cl . The effect of NH_4^+ thus differs considerably from that of the other monovalent cations tested.

Effect of Mg^{++}

Mg^{++} is known to decrease the birefringence of the intact muscle fibre (von Ebner, 1882) and the flow-birefringence of myosin solutions at a concentration of 0.35 M (Edsall and Mehl, 1940). In our experiments carried out in the micro-

TABLE V
Effect of Increasing Concentrations of NH_4^+ on the Δ of Myosin Solutions

NH_4^+ concentration	Δ at 500 R.P.M.
0 (control in 0.5 M LiCl)	27°
0.1 M (+ 0.4 M LiCl)	27°
0.5 M	17°
1.0 M	4°
2.0 M	0°

scope cell 0.05 M MgCl_2 decreased the Δ to 57 per cent of the control (myosin in 0.5 M LiCl). In the coaxial viscosimeter the flow-birefringence decreased steadily with rising concentrations of Mg ; the solution was nearly non-birefringent at a concentration of 0.225 M. The η/η_0 first increased with rising concentration of Mg^{++} and reached a maximum at 0.05 M; further additions of MgCl_2 decreased the relative viscosity (see Fig. 3). Anomalous flow was retained up to the highest of these concentrations. In another experiment (IV 195) the flow-birefringence reached zero value at 0.5 M, and the maximum relative viscosity was observed at 0.075 M.

Effect of Ca^{++}

The effect of Ca^{++} is somewhat similar to that of Mg^{++} . 0.05 M CaCl_2 reduces the Δ (measured in the microscope cell) to 65 per cent of the control, whilst 0.02 M had no effect. In the coaxial viscosimeter increasing concentrations of Ca^{++} (up to 0.4 M) produce a steady fall of Δ to zero, and a steady rise of relative viscosity (see Fig. 4).

In other experiments the same relationship was always found though the rate of rise of relative viscosity differed according to the myosin preparation used.

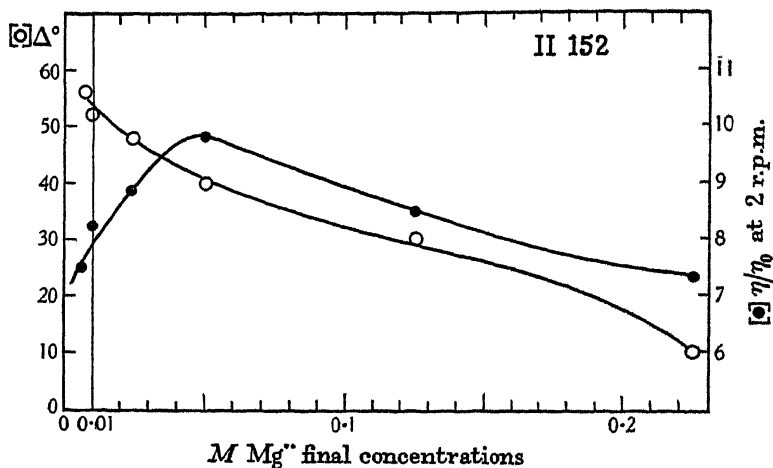


FIG. 3. Effect of Mg^{++} on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 0.67 per cent (Experiment II 152).

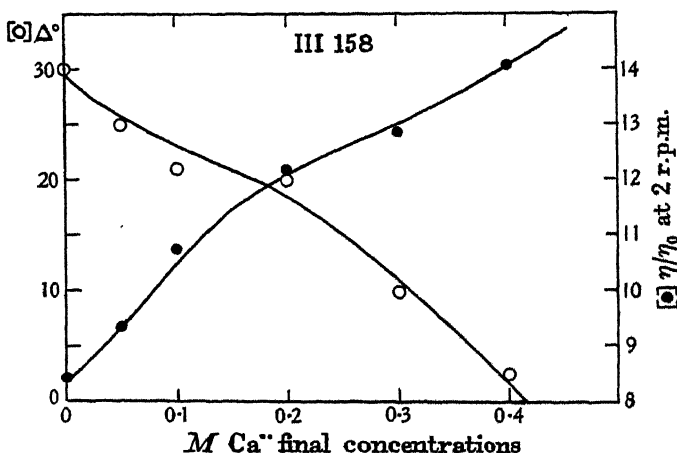


FIG. 4. Effect of Ca^{++} on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 1.15 per cent (Experiment III 158).

Effect of Anions

It is known that anions have a considerable effect on the physicochemical properties of colloidal solutions (Hofmeister's series). In our experiments the effects of 0.5 M KCl, KBr, KI, KSCN, and K_2SO_4 on the Δ and η/η_0 were compared. Equal amounts of myosin were dissolved in equimolecular amounts

of the above salts and Δ and η/η_0 were measured in the coaxial viscosimeter. As shown in Table VI, Br^- and SO_4^{2-} have no appreciable effect on the Δ of myosin as compared with Cl^- , whilst I^- and SCN^- abolish, or in some experiments, considerably reduce the Δ . The relative viscosity also is unaffected by Br^- and SO_4^{2-} as compared with Cl^- . I^- was found to reduce the η/η_0 , but the values increase with time and within 30 minutes after the first measurements the values of η/η_0 are nearly as high as, or exceed those of the control. SCN^- generally increases the relative viscosity of myosin solutions. In one of our experiments (Experiment III 175) an increase of about 200 per cent was observed (η/η_0 in control 8.5, in KSCN 28.5). In the experiment quoted in Table VI, however, no appreciable effect of SCN^- on the viscosity of myosin was observed.

TABLE VI
Effect of 0.5 M Anions on the Flow-Birefringence and Viscosity of Myosin
Experiment III 189. Final concentration of myosin 1.86 per cent protein.

Electrolyte	Δ at 50 R.P.M.	η/η_0 at 2 R.P.M.
KCl.....	70°	8.0
KBr.....	65°	7.3
KI.....	0° first reading	4.6
	after 15 min.	5.4
	" 30 "	5.8
KSCN.....	0°	7.7
K ₂ SO ₄	70°	7.7

Effect of pH

Edsall and Mehl (1940) mentioned that variations of pH between 6-8 have no effect on the Δ of myosin, whilst they show that higher pH (9-11) irreversibly reduces the Δ . In our experiments the effect of pH on Δ and η/η_0 was examined on the same myosin solution in the coaxial viscosimeter. Equal amounts of myosin dissolved in 0.5 M KCl were mixed with phosphate buffer of varying pH. The final concentration was 0.1 M phosphate buffer. The pH of the myosin solutions was measured electrometrically.

As shown in Fig. 5 (result of four experiments) variations of pH between 6.0 and 8.5 have no appreciable effect on the Δ of myosin, thus confirming the results of Edsall and Mehl. If the pH is lowered below 6.0 a sudden increase of Δ is observed, simultaneous with an appearance of turbidity of the myosin solution. At pH 5.4-5.5 the myosin solution gels. It is therefore possible that the observed increase of Δ is due to a photoelastic effect of shear stress on myosin precipitating near the isoelectric point rather than to a genuine increase in flow-birefringence. At alkaline ranges Δ falls off steadily as the pH

increases from pH 8.5–11. This had early been noted (von Muralt and Edsall, 1930, p. 376; Edsall and Mehl, 1940), and it has, of course, long been known that the birefringence of the intact muscle fibre suffers a great, though reversible, reduction, in the presence of alkali (Nasse, 1882; von Ebner, 1882).

The relative viscosity is very high at pH lower than 6.0, and falls off parallel to Δ with increasing pH; it does not change between pH 6–8.5. But contrary

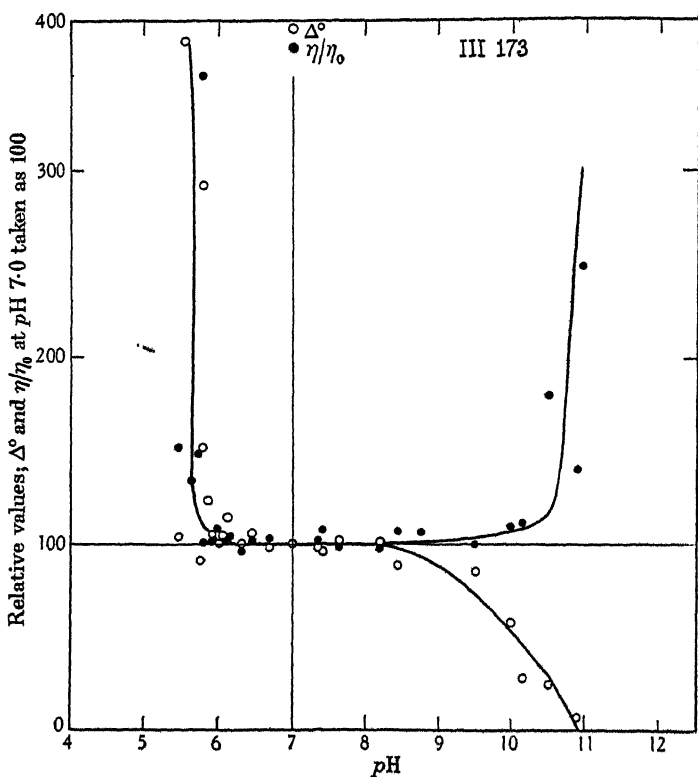


FIG. 5. Effect of pH on the flow-birefringence and relative viscosity of myosin (Experiments III 161–173).

to Δ the η/η_0 increases greatly with a further rise in pH. At the same time, above pH 10.5 the flow anomaly is lost.

It is interesting to contrast the behaviour of myosin at different pH with that of the other outstanding fibrillar corpuscular protein, TMD virus. According to Lauffer (1938 *b*) its Δ falls off with increasing pH and disappears between 9.5 and 10, but η/η_0 , instead of entering upon a marked rise, also falls off. Moreover, as the pH decreases there occurs about 5.5 a marked rise in η/η_0 as in the case of myosin, but this is not accompanied by a marked rise in Δ .

Effect of Urea

Urea is known to decrease the flow-birefringence of tobacco mosaic virus solutions (Bawden and Pirie, 1940) and of myosin (Edsall and Mehl, 1940). On the other hand, urea has been used as an extractant to confer flow-birefrin-

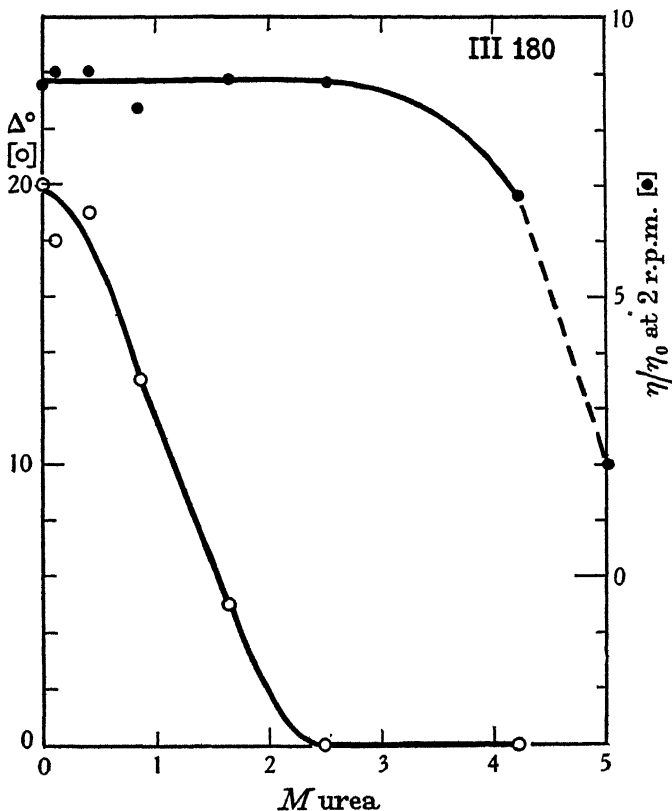


FIG. 6. Effect of increasing concentrations of urea on the flow-birefringence and relative viscosity of myosin. Final concentration of myosin 1.0 per cent (Experiment III 180).

gence on proteins from the liver and kidney (Banga and Szent-Györgyi, 1940; see Lawrence, Miall, Needham, and Shen, 1944). In our experiments the effect of increasing concentrations of urea on the Δ and η/η_0 of myosin solutions was studied. Varying amounts of urea were added to equal volumes of myosin dissolved in 0.5 M LiCl and Δ and η/η_0 were measured in the coaxial viscosimeter. As shown in Fig. 6, the flow-birefringence of myosin falls off when the concentration of urea is higher than 0.5 M, and reaches 0° at approximately 2.5 M.

The relative viscosity, however, is still unaffected at this concentration, and only higher concentrations (4.2 M) reduce it appreciably. The decrease of relative viscosity by high concentrations of urea had been already reported by Edsall and Mehl.

REVERSIBILITY OF THE REDUCTION OF FLOW-BIREFRINGENCE AND ANOMALOUS VISCOSITY

In view of the facts to be described below, the conception generally prevailing (cf. Edsall, 1942) that decreases in the flow-birefringence of myosin are irreversible and necessarily imply any far reaching denaturation, must now be abandoned. After the action of adenosinetriphosphate, as we shall see, Δ invariably returns to its initial value, and a variety of other effects which we encountered convinced us that this phenomenon does not stand alone. At the same time, we do not wish to suggest that the mechanism of the reversibility is in all cases the same.

Ageing and Temperature Effects.—If a flow-birefringent solution of myosin is allowed to stand at 0°C. for 10 days or more, under sterile conditions, it will often be found to have lost its flow-birefringence and its anomalous viscosity, while in all other characteristics it seems unaltered. We have found that if such a non-birefringent solution is placed for a short time at 37°C. it will rapidly become flow-birefringent again. If allowed to remain too long at 37°C. it will set to a thixotropic gel, as will any specimen of myosin if exposed to this temperature for more than about an hour. Fig. 7 (Experiment II 113) shows the restoration of flow-birefringence and anomalous viscosity to such an 8 day old solution in 0.5 M KCl; the flow-birefringence has risen after 2 hours at 37°C. from 0° to 55° Δ and bulk anomaly has returned. That this birefringence cannot be due to the photoelastic effect of a gel under strain is shown partly by the fact that the relative viscosity has risen very little, but more especially by the fact that under such conditions the angle of isocline is found to be unchanged. In another experiment of the same sort (II 118) the angle of isocline (ψ) was 52° before the heat treatment, and 54° after 2 hours at 37°C., indicating that the particles were still perfectly free to orient. At the same time Δ had risen from 20° to 60°. This evidence was all the more convincing because in this second experiment the relative viscosity rose rather more than in the first (η/η_0 before 2.02, after 3.15, at 7 R.P.M.).

Engelhardt and Ljubimova (1939), who studied the inactivation of adenosinetriphosphatase by heat, noticed that in the presence of its substrate, the enzyme was easily inactivated by temperatures such as 37°C. We accordingly made some observations on the effect of adenosinetriphosphate on the change in the physical properties of myosin at this temperature. From these figures several points emerge. In absence of ATP exposure to physiological temperature greatly increases the flow-birefringence, but without sensibly affecting the

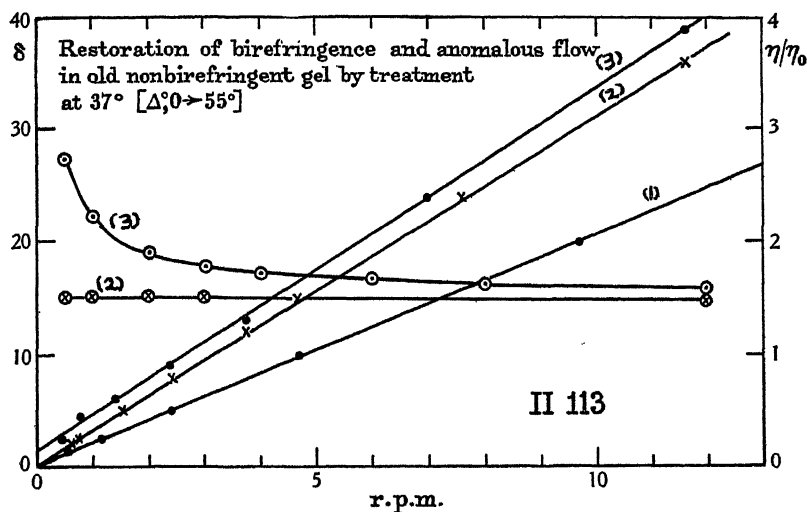


FIG. 7. Restoration of flow-birefringence and anomalous viscosity to an aged myosin sol by treatment at 37°C. (1) Curve for water; (2) curve for myosin before heating; (3) curve for myosin after heating 2 hours at 37°C.

TABLE VII
Protective Effect of Adenosinetriphosphate on Myosin at 37°C.

		Δ	ψ	$\frac{\eta}{\eta_0}$ at 3 R.P.M.	Flow anomaly
Experiment II 120	Myosin in 0.5 M KCl before	40	45	3.8	Marked
	Myosin in 0.5 M KCl after 2½ hrs. at 37°C.	45	48	—	—
	Myosin in 0.5 M KCl after 5 hrs. at 37°C.	102	54	>4.6	Extremely marked; viscosity measure- ments difficult
	Myosin in 0.5 M KCl + 0.1 M ATP	40	42	3.8	Marked
	Myosin in 0.5 M KCl + 0.1 M ATP after 2½ hrs. at 37°C.	35	47	—	—
	Myosin in 0.5 M KCl + 0.1 M ATP after 5 hrs. at 37°C.	62	52	2.1	Marked
Experiment II	Myosin in 0.75 M KCl before	55	47	1.41	Marked
	Myosin in 0.75 M KCl after 1 hr. at 37°C.	80	50	1.64	Very marked
	Myosin in 0.75 M KCl + 0.1 M ATP	55	41	1.41	Marked
	Myosin in 0.75 M KCl + 0.1 M ATP after 1 hr. at 37°C.	50	44	1.45	Marked

angle of isocline; hence here again the phenomenon cannot be one of photo-elastic gel strain. At the same time, the relative viscosity rises. In the presence of the substrate, however, these effects are inhibited; the flow-birefringence and relative viscosity do not rise to the same extent and the intensification of the flow anomaly is not observed. In all these experiments the enzyme was actively splitting the substrate (in Experiment II 120 0.213 mg. inorganic P appeared after $2\frac{1}{2}$ hours), and it is likely that when the substrate has been fully used up the usual physical changes will begin to take their course.

Treatment at 37° will also restore flow-birefringence after its abolition by various ions. Typical figures are quoted in Table VIII (Experiment ST 314). From this it can be seen that the particles of myosin, having either been disaggregated into smaller more spherical fragments or tangled together into

TABLE VIII
Restoration of Flow-Birefringence of Myosin after its Abolition by Ions, at 37°C .

	At the beginning of experiment (before additions) (320 R.F.M.)		After 4 hrs. at room temperature (20°C .) (320 R.F.M.)		After 1 hr. at 37°C . (320 R.F.M.)		Increase
	Δ°	ψ°	Δ°	ψ°	Δ°	ψ°	Δ°
All at 1.1 M; 0.5 M KCl + 0.6 M other ion							
Control KCl	50	82	50	82	80	72	30
LiCl	42	72	0	—	65	62	65
NH_4Cl	50	80	0	—	50	58	50
MgCl_2	50	80	16	—	55	62	39
CaCl_2	50	80	15	—	62	73	47

larger more spherical clumps, are restored by exposure to 37° to something approaching their original condition. It is interesting that the angle of isocline tends to be less than it was originally while the birefringence tends to be higher. This suggests a small reduction in axial ratio, combined with more regular intramolecular packing and hence higher intrinsic birefringence.

Spontaneous Restoration.—Apart from the spontaneous restorations of flow-birefringence always found after the action of adenosinetriphosphate, as described below, we have sometimes observed a similar effect if myosin is treated with low concentrations of urea or certain ions. Fig. 8 illustrates two such experiments (ST 315). With the preparation of myosin here used 2 M urea was not sufficient to produce a permanent abolition of the flow-birefringence, but it reduced it to two-fifths of its original value, while the angle of isocline correspondingly fell; then in a very short time both began to return, and by half an hour had reached their initial positions. Further additions of urea were

required to abolish permanently the flow-birefringence. The reversibility in this experiment is not so surprising, for we know from numerous observations (*e.g.* those of Bawden and Pirie, 1940, on virus nucleoprotein, and many other workers on other proteins) that the initial stages of urea denaturation are generally reversible; what is remarkable is its spontaneity. Such effects may possibly be relevant to the reversible action of adenosinetriphosphate, for which they may perhaps be regarded as models. Fig. 8 also shows a similar curve for myosin in potassium chloride solution acted upon by lithium chloride.

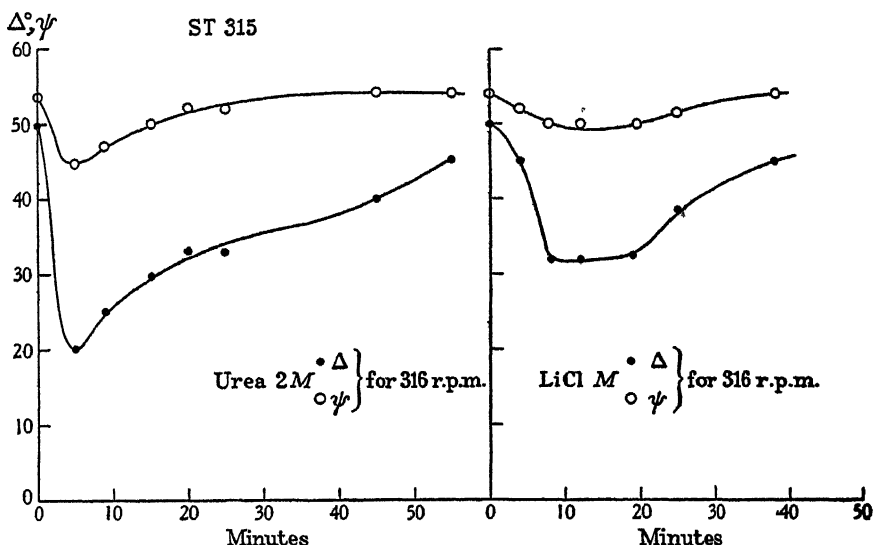


FIG. 8. Spontaneous restoration of flow-birefringence after treatment of a K^+ myosin sol with Li^+ and with urea.

These observations were extended by measurements in the coaxial viscosimeter (Experiment II 128). A myosin preparation showing Δ 65° ψ 54 (for 260 R.P.M.) was treated with 2 M urea; in 15 minutes its Δ had declined to 37° and ψ to 47° ; but by 80 minutes at $18^\circ C$. both values were back at their original positions. The myosin was then diluted four times with 2 M urea and examined in the coaxial viscosimeter. Its anomalous viscosity was considerably more pronounced than at the beginning of the experiment, and its relative viscosity had risen from 4.4 to 6.8 (at 3 R.P.M.). The preparation was then removed and boiled for 2 minutes, then re-examined in the coaxial viscosimeter. The treated protein, still fully in solution, now showed an anomalous viscosity and relative viscosity (4.4) exactly the same as that of the protein before urea had been added.

We do not wish at present to offer any explanation of the above observations, but it is sure that further work along these lines would be very profitable.

Reversibility by Removal of the Reduction Agent.—When the flow-birefringence of myosin solutions is reduced by addition of different electrolytes, a spontaneous return of Δ is not usually observed. If, however, the myosin solutions, the Δ of which has been reduced by addition of Mg^{++} or different concentrations of urea, are reprecipitated in water, the flow-birefringence returns. Experiments were set up as follows (Experiment app/349): to equal amounts of myosin dissolved in 0.5 M LiCl different salts and urea were added, and the Δ measured in the microscope cell. The myosin solutions were then precipitated by pouring them into ten volumes of ice cold distilled water; the precipitated myosin was collected by centrifuging, dissolved by addition of LiCl to the final concentration of 0.5 M, and the flow-birefringence measured again. As shown

TABLE IX
Restoration of Flow-Birefringence of Myosin by Removal of the Reduction Agents

Reduction agent	Δ in per cent of control	Δ in per cent of control after re-precipitation and re-solution
0.5 M LiCl (control).....	100	100
0.033 M adenosinetriphosphate.....	50	74
0.1 M $MgCl_2$	39	70
0.1 M urea.....	78	100
1.0 M urea.....	42	74
2.0 M urea.....	22	65

in Table IX, the flow-birefringence returned to a considerable extent, in some cases to the same extent as the controls. After 6.0 M urea, however, neither solubility nor flow-birefringence of the myosin were regained.

THE ADENOSINETRIPHOSPHATE EFFECT

If a myosin solution containing from 1 to 3 per cent of the protein is treated with adenosinetriphosphate at concentrations as low as 0.004 M, the flow-birefringence (Δ) is reduced at once by some 40 to 60 per cent (mean fall from Table X 48 per cent), the flow anomaly is unaffected, and the relative viscosity (η/η_0) is reduced by some 14 per cent. The subsequent return of both flow-birefringence and relative viscosity to their original values, which may take from 15 minutes to several hours, according to the conditions, is accompanied by the splitting off of inorganic phosphate from the ATP. The fall of flow-birefringence and relative viscosity is then exactly repeatable upon the same sample of myosin. The various features of this striking phenomenon will be taken up in turn in what follows.

TABLE X

Action of Adenosinetriphosphate and Inosinetriphosphate on the Flow-Birefringence of Myosin

Experiment No.	Conditions				Δ°	$\Delta^\circ_{\text{atp}}$	Fall of Δ°	Fall per cent of initial Δ	Time required for return of Δ
	Temperature	Reduction agent	Solvent	Method for determination of Δ°					
Once precipitated myosin									
	$^\circ\text{C.}$								hrs. min.
app/327	20	NaATP	KCl	mc350	43	15	28	65.1	19 30
app/328	20	NaATP	KCl	mc380	67	45	22	32.9	— —
app/329	20	KATP	KCl	mc380	68	42	26	38.4	— —
app/330	20	KATP	KCl	mc380	75	57	18	24.1	— —
app/332	37	NaATP	KCl	mc380	55	30	25	54.5	3 0
app/333a	37	KATP	KCl	mc380	48	27	21	43.8	3 10
app/333b	30	KATP	KCl	mc380	45	22	23	51.2	4 30
app/338	20	KATP	KCl	mc380	73	52	21	28.8	— —
app/339	37	KATP	KCl	mc380	37	23	14	38.0	2 0
app/341	20	KATP	KCl	mc380	28	15	13	46.5	— —
app/344 (dos)	20	KATP	KCl	mc560	75	20	55	73.5	>24 0
app/345 (P)	20	KATP	LiCl	mc570	50	25	25	50.0	20 0
app/350 (s 1)	20	NaATP	NaCl	mc514	65	25	40	61.5	— —
app/351 (s 2)	20	NaATP	NaCl	mc514	67	35	32	47.8	1 30
		NaITP	NaCl	mc514	67	44	23	34.3	0 15
app/352 (s 3)	20	NaATP	NaCl	mc514	64	37	27	42.3	— —
app/358	20	NaATP(KS)	NaCl	mc500	63	40	23	36.5	8 30
app/360	37	KATP	KCl	mc355	45	23	22	49.0	1 30
app/361	37	KATP	KCl	mc385	62	47	15	22.4	2 10
app/363	20	KATP	LiCl	mc204	75	40	35	46.8	— —
II 111	20	KATP	KCl	mc316	45	28	17	37.8	— —
II 141	20	KATP	LiCl	cv 50	62	22	40	64.5	0 45
II 143	20	KATP	LiCl	cv 50	37	7	30	81.1	3 15
II 148	20	KATP	LiCl	cv 50	52	24	18	53.9	2 30
III 176	20	KATP	LiCl	cv 50	48	17	31	64.8	1 55
				cv 50	48	25	23	48.0	1 0
				cv 50	48	22	26	54.1	1 0
Thrice precipitated myosin									
app/363(s 4)20		CaATP	NaCl	mc514	45	28	17	37.0	0 40
		NaITP	NaCl	mc514	45	25	20	44.5	0 40
		CaITP	NaCl	mc514	45	38	7	15.5	0 20
app/355(s 5)20		NaATP	NaCl	mc514	76	55	21	27.8	0 6
app/356(d 1)20		NaATP	NaCl	mc514	92	79	13	14.1	0 10

 Δ° , flow-birefringence
mc, microscope cell Δ° , initial
cv, viscosimeter $\Delta^\circ_{\text{atp}}$ after ATP

The immediate reduction of flow-birefringence when myosin and adenosine-triphosphate are brought together is illustrated in the curves of Fig. 9 (Experiment app/329)—a typical experiment, though the reduction is frequently greater. In order to avoid all ionic effects, K^+ or Na^+ was generally the only cation present, but as can be seen by inspection of Table X, various combinations of cations do not obscure the phenomenon. From the data already given in earlier sections of this paper, it is obvious that strict control of the salt con-

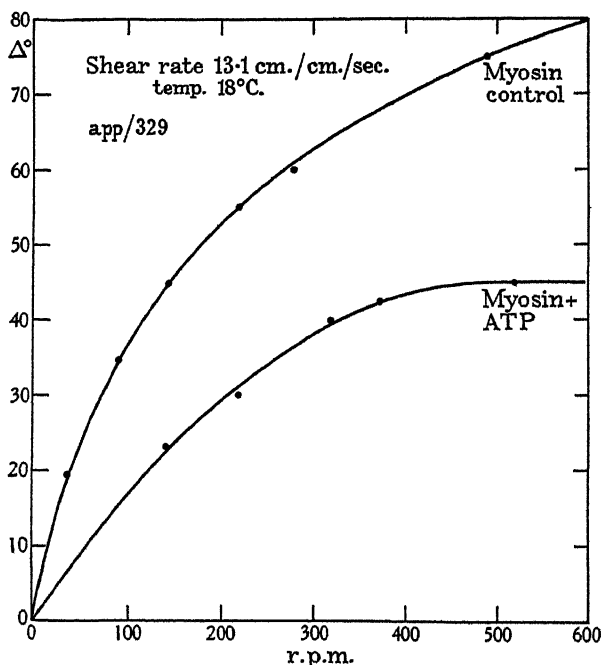


FIG. 9. Reduction of the flow-birefringence of myosin by addition of adenosine-triphosphate.

centration is essential, since the flow-birefringence of myosin varies with the molarity of the salt solution in which the protein is dissolved. Hence the ionic strength of the adenosinetriphosphate or other substances added was always compensated for in such a way as to bring all samples to a final concentration equivalent to a known ionic strength.

Close attention was paid to the stoichiometric aspect of the phenomenon. When the concentration of adenosinetriphosphate is varied at 20°C. and neutral pH, it is found that for a myosin solution of 0.86 per cent protein, approximately the full effect is obtained at 0.004 M, falling off very rapidly at lower concentrations, and being very little higher in amounts up to 0.2 M. These

relationships are illustrated in Fig. 10 (Experiment app/344). It should be emphasised that this concentration is much lower than that of any of the other classes of substances which affect myosin's flow-birefringence. To produce a

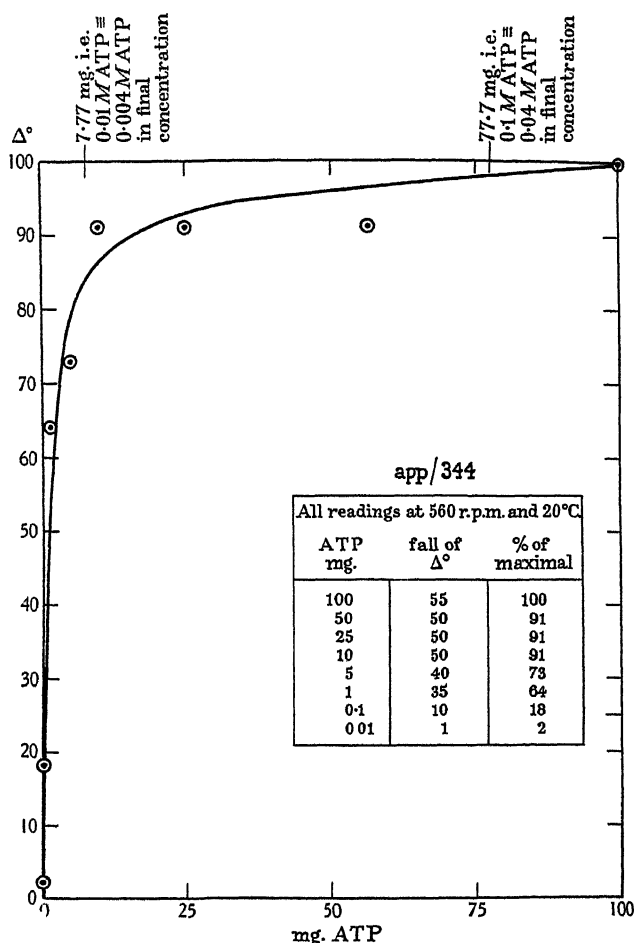


FIG. 10. Relation of myosin flow-birefringence reduction effect to concentration of adenosinetriphosphate.

60 per cent fall of flow-birefringence, 1.5 M urea is required, 1.0 M monovalent cation, and 0.2 M divalent cation; or a pH of 10. Moreover, concentrations of adenosinetriphosphate as low as 5.10^{-5} M will bring about a fall of flow-birefringence of some 15 per cent of the maximum fall.

The temperature coefficient of the fall of flow-birefringence of myosin caused by adenosinetriphosphate is a high one. In the experiment just quoted, at

the highest ATP concentration, the flow-birefringence fell from $\Delta 75^\circ$ to $\Delta 25^\circ$ within 5 minutes from the moment of mixing, and in other experiments we always found that the fall goes to completion in under 5 minutes. Such are the figures for room temperature ($16-20^\circ\text{C}.$), but if the experiment is carried out at $0^\circ\text{C}.$ then the fall is greatly prolonged. Fig. 11 (Experiment app/335) illustrates such an experiment. Since the temperature coefficient is thus appropriate for a chemical reaction, we have adopted the working hypothesis that the birefringence reduction signifies the union of adenosinetriphosphatase with its substrate.

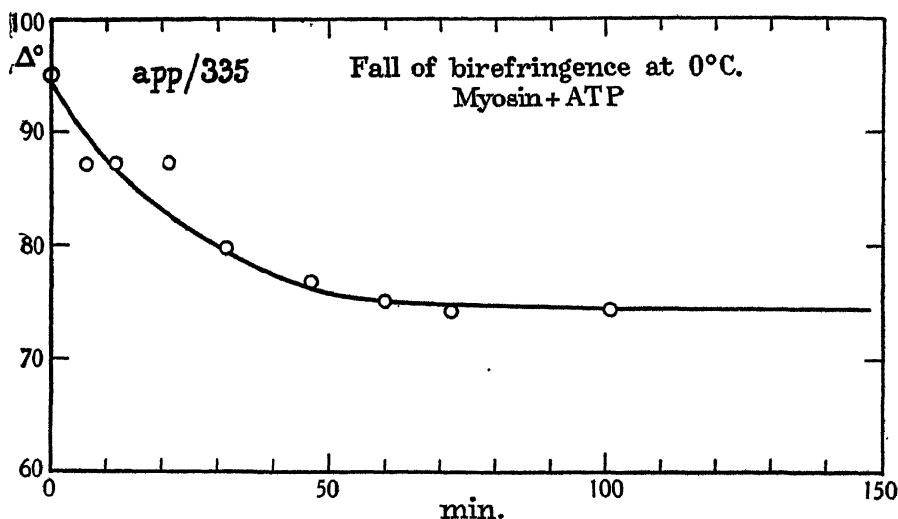


FIG. 11. Fall of flow-birefringence at $0^\circ\text{C}.$

The time required for the complete spontaneous return of the flow-birefringence of myosin to its original value before the addition of adenosinetriphosphate varied considerably in different experiments (see Table X). It was much shortened by allowing the phosphorolysis of ATP to proceed at $37^\circ\text{C}.$ instead of $20^\circ\text{C}.$ Especially notable is the fact that with thrice precipitated myosin the return time of Δ is extremely short, *i.e.* less than three-quarters of an hour as compared with the 1 to 3 hours taken by once precipitated myosin at 37° and the 20 hours or so at $20^\circ\text{C}.$ This behaviour is readily explained by the fact that myosin, unless purified by repeated precipitations, contains the myokinase of Kalckar (1942), which converts two molecules of ADP to one of ATP and one of adenylic acid. In presence of this agent, then, there will be a continuous, if slow, formation of adenosinetriphosphate, which will provide further quotas of substrate for the myosin as the breakdown of the ATP

originally added goes on. So rapid, indeed, is the return in the absence of myokinase that it is difficult to obtain the lowest point reached by Δ immediately after mixing, and the per cent falls appear therefore to be less for thrice precipitated myosin samples than for the rest.

At the same time as the fall in flow-birefringence, there occurs a marked change in the viscosity properties of the myosin sol. While the anomaly of

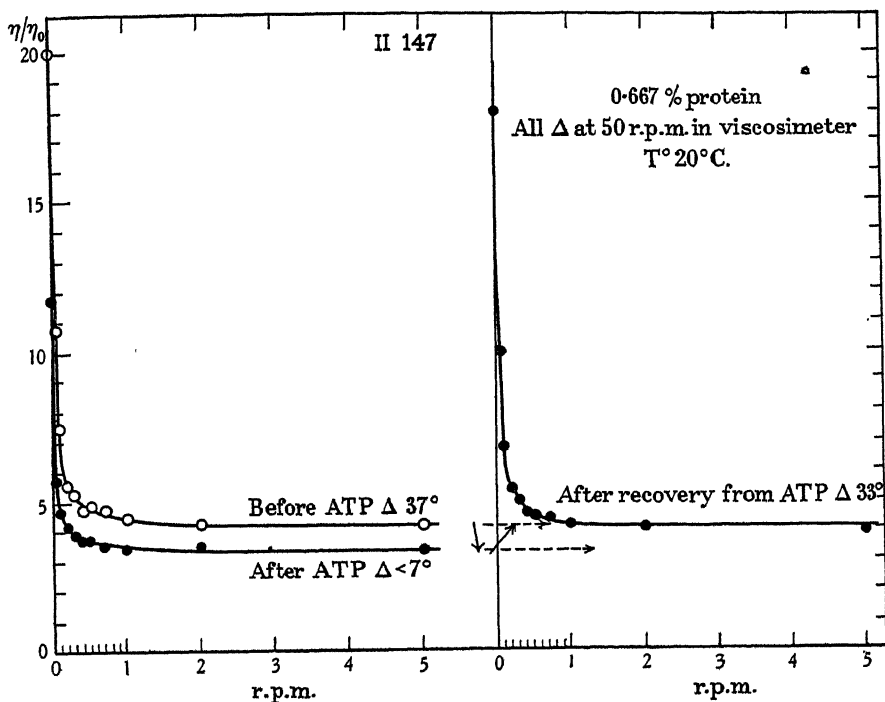


FIG. 12. Retention of flow anomaly and reversible reduction of the relative viscosity of myosin by addition of adenosinetriphosphate.

flow is not appreciably changed, the relative viscosity declines some 14 per cent—*cf.* Fig. 12 (Experiment II 147) in which η/η_0 falls 20 per cent, from 4.4 to 3.5 at 1 R.P.M. upon the addition of adenosinetriphosphate, rising again spontaneously to 4.3 when recovery to the initial flow-birefringence has almost been completed, and no further ATP remains to be split. This is a typical experiment out of many in which flow-birefringence and viscosity properties were measured upon the same sample of myosin in the coaxial viscosimeter, though necessarily at different rates of shear.

Among the most demonstrative of such experiments, we may give one in

which three successive falls and returns of flow-birefringence were observed in the same sample of myosin upon three successive additions of adenosinetri-

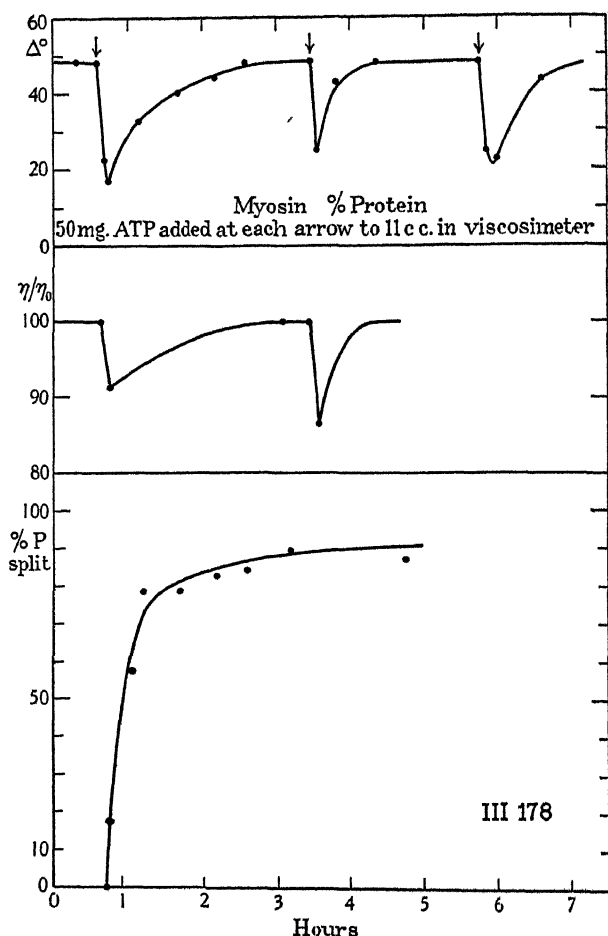


FIG. 13. Three successive falls and recoveries of flow-birefringence of a myosin sol treated three times with adenosinetriphosphate, with two successive falls and recoveries of relative viscosity (expressed in percentage of initial value) and estimations of inorganic phosphate liberated by adenosinetriphosphatase activity of the myosin during the first cycle.

phosphate, with two successive falls and returns of relative viscosity, while over the first period the splitting-off of inorganic phosphate was measured. As in many other experiments, the phosphorolysis proceeded normally in the viscosimeter while readings of the protein's physical properties were being taken. From Fig. 13 (Experiment III 178) and Table XI it may be seen that

the first fall of Δ was 64 per cent, the second 48 per cent, while the third was 54 per cent. The first viscosity fall was just under 10 per cent and the second just under 15 per cent. It was noticeable here—as also in several other experiments of the same kind—that the curve of the time relationship of the splitting of ATP does not agree over its whole course with the curves of re-

TABLE XI

Action of Adenosinetriphosphate upon the Relative Viscosity and Flow-Birefringence of Myosin. Temperature 20°C.

Experiment No.	Conditions		Protein concentrations	η/η_0 at 1 R.P.M.			Ratio η_i/η_{atp}	f			R.P.M. used for Δ	Δ°		$(\eta_g - \eta_0) \times 10^6$	Ratio Δ_i/Δ_{atp}
	Solvent	Reduction agent		Initial	atp.	Recovery		Initial	atp.	Initial		atp.			
II 111	KCl	KATP	0.078	1.79	1.67	Not measured	1.07	62.0	57.5	316	45	28	148	87	1.70
II 147	LiCl	KATP	0.667	4.4	3.5	4.3	1.25	45.0	39.0	50	37	7	121	24	5.05
II 149	LiCl	KATP	0.932	10.4	7.6	10.8	1.37	63.5	53.0	50	52	24	170	78	2.19
II 141	LiCl	KATP	1.01	11.8	11.0	Not measured	1.07	66.0	63.0	50	62	22	204	72	2.82
III 178a	LiCl	KATP	1.33	18.8	17.4	19.0	1.08	73.0	70.0	50	48	17	158	56	2.82
III 178b	LiCl	KATP	1.33	26.6	23.0	29.5	1.16	87.7	81.0	50	48	25	158	83	1.90

f , length/ratio of particles

η/η_0 , relative viscosity

η_i , relative viscosity, initial

η_{atp} , relative viscosity, after ATP

Δ° , observed extinction angle

Δ_i , observed extinction angle, initial

Δ_{atp} , observed extinction angle after ATP

$n_e - n_0$, birefringence

turn of flow-birefringence and relative viscosity. Interpretation of this fact must await further investigation; more than enough ATP may have been present at the beginning than was required to saturate the enzyme.

When our relevant data for relative viscosity are summarised and compared with the simultaneously measured flow-birefringence—as in Table XI—a further perspective is gained. The experiments are arranged in order of increasing protein concentration over a nearly twentyfold range, but it is seen that the ratios of falls of relative viscosity and flow-birefringence are more or less independent of conditions such as protein concentration and ions present.

The axial ratio of the particles, f , has been calculated according to the simplified formula of Robinson (1939):

$$\eta/\eta_0 = 1 + \frac{Vf^2}{4}$$

where V is the volume fraction in gm. (cc.)/cc., and f is the length/radius of the particle (see Lawrence, Needham, and Shen, 1944, p. 208). The birefringence has also been calculated from the observed extinction angles (Δ°).

The interesting point which the table presents is that the decrease in axial ratio of the particles caused by ATP, is not, as judged from viscosimetry alone, very large. The η/η_{atp} ratio is of the order of 1.1. But the corresponding measurements of flow-birefringence show a much larger fall, the $\Delta_i/\Delta_{\text{atp}}$ ratio being of the order of 2.7.

At least two possible explanations are available for this. It must be remembered that the birefringence measurements are all taken *perforce* at considerably higher shear rates than the viscosimetric measurements. On the one hand, then, we may accept the hypothesis of sliding parallel extension and retraction in the micelle, as given by Lawrence, Needham, and Shen (1944, p. 228), and assume that no marked changes take place in intermicellar forces. In this case, the main contractive effect of ATP would be on the parallel sliding motion (whether or not exerted in conjunction with a keratin-like configurational folding process) and this would obviously manifest itself much more markedly on the high shear rate birefringence properties than upon the low shear rate viscosity properties. The pulled-out anisometric micelles would contract, and hence the flow-birefringence would drop, but they would remain anisometric micelles, and hence the relative viscosity would remain fairly high. Thus about three-quarters of the ATP effect would be on the parallel sliding system, and one-quarter of it would be on the closed micelle itself. The former might be called an "anti-plasticiser" effect, and the latter might be the configurational effect.⁴

An alternative interpretation would assume that some factor, probably connected with changes in intermicellar forces, is masking the fall in relative viscosity which the solution should, according to its flow-birefringence, show. This factor also might not be without physiological significance.

Lastly, it will be noticed from Table XI that the axial ratio of the myosin particles before the addition of ATP is in rough agreement with values in the literature now widely accepted. Our average figure makes them 31.7 times as long as they are broad; this may be compared with a figure from x-ray analysis

⁴ In practice, the ATP was always added when the sol was at rest. Hence the 14 per cent effect on viscosity would appear to be a true contraction while the 48 per cent effect on birefringence would appear to be an inhibition of the pulling-out effect otherwise seen at the higher shear rates.

of 27.3 (2050 Å long, 75 Å broad) given by Worschitz (1935) although in the present state of our knowledge such an agreement may be but fortuitous. Compare also the axial ratio estimates for TMD virus of 36.8 (Frampton and Neurath, 1938), 35 (Lauffer, 1938a), and 55 (Neurath and Saum, 1938).

From the observations just summarised, the question immediately arises whether we have to deal here with a true contractility of the myosin molecules in the sol, analogous to the reversible configuration changes in keratin and myosin which have been revealed by the x-ray techniques. Data such as those of Fig. 12 do, after all, recall the classical twitch diagrams of intact muscles. Since the flow anomaly is retained, the particles must clearly continue to be rod-like or fibrillar, but since the flow-birefringence is reduced, their axial ratio must be less than before. This view is supported by the fact that the angle of isocline also decreases slightly, as shown in a few typical figures collected in Table XII, and returns again to its original value when the

TABLE XII
Action of Adenosinetriphosphate upon the Angle of Isocline of Myosin

Experiment No.	ψ_i	ψ_{atp}
app/329	62	52
app/330	58	52
app/341	66	64
app/363	76	67

flow-birefringence likewise returns. Von Muralt and Edsall's figure (1930) for ψ on normal myosin preparations was around 70°. The decrease in relative viscosity would then be expected, since we know from work on polymers (Kraemer and van Natta, 1932; Powell and Eyring, 1942; Mark and Simha, 1940) that for highly asymmetric molecules, the relative viscosity is proportional to the polymer length or axial ratio at equal molar concentrations.

At the same time, in the absence of some reliable estimate of the number of particles present before and after the treatment with adenosinetriphosphate, changes of aggregation and even of hydration are also conceivable. Rod-like particles might split transversely so that their axial ratio would greatly decrease. The perfection of the spontaneous return of the physical properties after the accomplishment of the enzyme action, however, seems to argue against disaggregation of the myosin micelles or molecules. But even if the phenomenon were wholly one of reversible disaggregation, rather than of true contraction and extension, the physiological significance of the effect might probably be none the less.

There is, moreover, the third possibility, namely intramolecular configurational

changes. In the first paper of this series (Lawrence, Needham, and Shen, 1944), it was pointed out that myosin molecules in a myosin micelle may be free to move one upon another like the opening and closing of slide-rules. Adenosinetriphosphate might then have an inhibitory effect on the opening.

From the stoichiometric data given above, a tentative calculation may be attempted, adopting the figures of Astbury and Bell (1941), including an equivalent weight of 67,000 for myosin, that 30 molecules of adenosinetriphosphate are required for every molecule of myosin, or one for every 19 amino acids in the myosin chain. Whether the fact that arginine occurs 32 times in the repeat unit, or once for 18 amino acids, may have any significance in this connection, remains for further consideration. But the possibility now arises that adenosinetriphosphate, the actual energy-providing substrate of the contractile protein itself, may in fact be the agent of contraction, which it would bring about by its initial combination with the enzyme.

Following this train of thought, it would be desirable to have experiments designed to show that if the enzyme-substrate combination is inhibited no change in flow-birefringence occurs; and if the subsequent enzymic action is inhibited, no restoration of the original flow-birefringence occurs. As will be seen below, we have obtained much evidence that the fall of flow-birefringence and the enzyme action may be inhibited together by substances which would be expected to be competitive inhibitors for the enzyme's normal substrate. We also carried out a few experiments, however, in which attempts were made to inactivate the enzyme by exposure to 37°C. before the substrate was added. These were not very clear cut because it is difficult to inactivate the enzyme-protein completely without bringing it to the thixotropic gel stage; however, in one case (Experiment app/337) after myosin had been held at 37°C. for 20 minutes before the addition of ATP, the fall of Δ was reduced from 50 per cent to 26.5 per cent, the return to the original value was completed in 1 hour, and the phosphorolysis inhibited 62 per cent. Since we know (D. M. Needham, 1942; Bailey, 1942) that Ca^{++} greatly activates adenosinetriphosphatase, a better way of inactivating the enzyme would be to free it entirely from this ion, perhaps by dissolving it as a sol in oxalate and vigorously centrifuging, but we have not so far succeeded in experiments along this line.⁵ It will, however, be seen from Table X that the return time with CaITP is shorter than with NaITP .

A control of some importance which may find mention at this point is that of the refractive index of the medium. Flow-birefringence may be caused either by orientation in stream lines of rods or plates themselves intrinsically bire-

⁵ According to a private communication from Dr. Bailey oxalate does not prevent splitting of ATP, when added to Ca myosin. Probably calcium oxalate is fairly soluble in presence of protein.

fringent, or by such orientation of rods or plates themselves isotropic if they are suspended in a medium of different refractive index to themselves. It was therefore necessary to check the refractive indices of solutions of ATP and myosin at different salt concentrations. As will be seen from the accompanying figures, the differences are very small and cannot account for the 50 per cent fall in Δ which myosin suffers when in the presence of adenosinetriphosphate.

	Refractive index at 17.2°C.
Distilled water.....	1.3335
0.5 M KCl.....	1.33855
1.0 M KCl.....	1.3435
KATP 50 mg./cc. molarity equivalent 0.5 M KCl.....	1.34015
KATP 100 mg./cc. molarity equivalent 0.625 M KCl.....	1.3437
Myosin 1 per cent in 0.5 M KCl.....	1.3420
Myosin 1 per cent in 0.95 M KCl.....	1.3460
Myosin 1 per cent in 1.43 M KCl.....	1.3545

In Stübel's experiment (1923) a range of refractive index of from 1.3 to 1.7 was shown to be necessary to bring about marked changes in the birefringence of the intact muscle fibre.

PROTEIN SPECIFICITY OF ADENOSINETRIPHOSPHATE

The question may be asked whether adenosinetriphosphate might possibly affect other fibrous corpuscular proteins in the same way as myosin. The only other member of this group with which we have so far worked is the virus nucleoprotein of tobacco mosaic disease, and we therefore tested the action of adenosinetriphosphate on its flow-birefringence. No fall of flow-birefringence was observed.

SUBSTRATE SPECIFICITY OF MYOSIN

On approaching this subject we should like to emphasise that the viscosity data themselves indicated a certain specificity in the action of adenosinetriphosphate on myosin. With cations and at alkaline pH ranges Δ falls while η/η_0 rises; with urea Δ falls while η/η_0 remains constant; only with adenosinetriphosphate do Δ and η/η_0 fall together to a limited and well defined extent, which larger doses will not increase, and which is spontaneously reversed during the enzyme action. It therefore seemed important to explore more thoroughly the action of other substances which might have the same effect as adenosinetriphosphate.

A number of intermediate compounds in carbohydrate breakdown and substances related to them were examined. The investigations were carried out

in the microscope cell by comparing the effect of 0.03 M solutions of the substances (final concentration) on myosin solutions in 0.5 M NaCl or LiCl with that of an equimolecular amount of ATP and with a control without any additions. The following substances had no effect on the Δ of once precipitated myosin solutions: lactate, glucose, glucose-*L*-phosphate (Cori ester), hexose-6-phosphate (Embden ester), hexosediphosphate (Harden-Young ester), glycerophosphate, phosphoglycerate, creatinephosphate, adenylic acid, inosinic acid, aneurin, aneurin-pyrophosphate (cocarboxylase), diphenylpyrophosphate (Neuberg and Wagner, 1926), inorganic pyrophosphate, inorganic triphosphate, metaphosphate, hexametaphosphate.⁶

TABLE XIII

Action of Adenosinediphosphate and Inosinetriphosphate on the Flow-Birefringence of Myosin

Experiment No.	No. of myosin precipitations	Final concentration of protein	Substrate 0.03 M	Δ at 514 m. μ
		<i>per cent</i>		
app/351	1	3.58	Control	67°
			Adenosinetriphosphate	35°
			Adenylic acid	62°
			Adenosinediphosphate	46°
			Inosinic acid	65°
			Inosinetriphosphate	44°
app/353	3	2.29	Control	45°
			Adenosinetriphosphate	27°
			Adenosinediphosphate	46°
			Inosinetriphosphate	25°

The only substances which we have so far found to affect the Δ of once precipitated myosin solutions to an extent comparable with ATP are adenosinediphosphate and inosinetriphosphate (see Table XIII). The former compound, however, has no effect on the Δ of thrice precipitated myosin. It may therefore be concluded that the effect of ADP is due to the formation of ATP from the diphosphate by myokinase (Kalckar, 1942).

Myosin and Sodium Triphosphate.—Although the sodium triphosphate was without effect on the Δ of myosin solutions, the observations of Neuberg and Fischer (1937) on the formation of orthophosphate from inorganic triphosphate by muscle and kidney extracts invited a further investigation of the effect of myosin on this salt.

Experiments in which myosin and $\text{Na}_5\text{P}_3\text{O}_{10} \cdot 6 \text{H}_2\text{O}$ were incubated at 37° and

⁶ In view of the fact that certain drugs are believed to have a direct effect on the muscle cell, we also examined the action of varying concentrations of acetylcholine, adrenalin, veratrine, nicotine, and iodoacetate on the flow-birefringence of myosin. No effects on Δ were observed, (*cf.* the work of Mirsky, 1938).

20° show that the inorganic triphosphate is enzymatically split by myosin although at a slower rate than ATP (see Tables XIV and XVII).⁷

The extent to which PO_4^{4-} is formed from $\text{Na}_5\text{P}_3\text{O}_{10}$ in the presence of myosin was examined in the following way: thrice precipitated myosin was incubated at 37°C. with Na triphosphate and CaCl_2 (which is known to activate the splitting of ATP by myosin; Szent-Györgyi and Banga, 1941; D. M. Needham, 1942; Bailey, 1942); the whole volume being adjusted to 0.5 M NaCl. Samples were withdrawn at intervals, the myosin precipitated by addition of 4 per cent trichloroacetic acid, and inorganic P was estimated in an aliquot of the filtrate. A control in which the myosin was replaced by distilled water was made in each case. It was shown in one experiment that a mixture containing Ca^{++} but otherwise identical with a control showed twice the amount of inorganic P split from added $\text{Na}_5\text{P}_3\text{O}_{10}$ in the same time (see Table XV,

TABLE XIV

Enzymatic Splitting of Sodium Triphosphate by Myosin

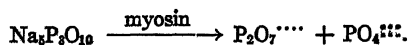
Final concentration of myosin 3.58 per cent protein; volume in tube adjusted to 3.0 ml. 0.5 M NaCl; incubated 1 hour.

Additions (final concentration)	Temperature	Inorganic P found	Inorganic P formed
	°C.	mg.	mg.
0.025 M sodium triphosphate.....	20	0.298	
0.025 M sodium triphosphate + myosin.....	20	0.396	0.098
0.025 M sodium triphosphate.....	37	0.363	
0.025 M sodium triphosphate + myosin.....	37	1.134	0.771

Experiment 1). As shown in Table XIV (Experiment 2), about 28.4 per cent of the total P in $\text{Na}_5\text{P}_3\text{O}_{10}$ is split off as PO_4^{4-} . No inorganic P was found in controls where inorganic triphosphate was incubated without myosin.

It should be noted that acids split sodium triphosphate to pyrophosphate and metaphosphate; the latter is then slowly converted at room temperature into orthophosphate. All the inorganic P estimations were therefore carried out at the end of the experiment as rapidly as possible, the deproteinised samples being kept in ice.

Since it was shown in a number of experiments that 22 to 29 per cent of the total P of added $\text{Na}_5\text{P}_3\text{O}_{10}$ appears as orthophosphate when incubated to completion of the reaction with myosin, the reaction would appear to be:



⁷ Dr. Bailey (private communication) found no splitting of Na triphosphate by very low concentrations of myosin, although these readily split ATP. Our observation may therefore be due to another enzyme.

Attempts were made to separate sodium pyrophosphate crystals from the incubation mixture, since the properties of the inorganic phosphates are so similar that a chemical separation of pyrophosphate appeared to be cumbersome. Although experiments using as much as 0.5 gm. of the sodium triphosphate were made, the results were inconclusive, chiefly due to the large amount of NaCl which it is necessary to have present and which also crystallises out.

Competitive Inhibition of the Effect of ATP on Myosin Solutions.—The investigation of the effect of ATP on the physical properties of myosin solutions suggests that it may be due to enzyme-substrate combination (*cf.* p. 384).

TABLE XV

Effect of Ca^{++} on the Enzymatic Splitting of Sodium Triphosphate by Myosin

Final concentration: myosin 1.85 per cent protein; 0.01 M $\text{Na}_5\text{P}_3\text{O}_{10}$; 0.5 M NaCl. Incubated at 37°.

Experiment No.	Time	Addition	Inorganic P found
	<i>min.</i>		<i>mg.</i>
1	0	0	Trace
	30	0	0.315
	60	0	0.562
	0	0.0045 M CaCl_2	0.270
	30	0.0045 M CaCl_2	0.720
	60	0.0045 M CaCl_2	1.282
2	0	0.0045 M CaCl_2	0.278
	10	0.0045 M CaCl_2	1.110
	20	0.0045 M CaCl_2	2.10
	30	0.0045 M CaCl_2	2.18
	60	0.0045 M CaCl_2	2.64

If this were so, any related substances which were competitive inhibitors for the enzyme action should also block the fall of Δ produced by ATP.

Experiments set up to investigate this problem were carried out as follows: To a set of tubes containing myosin solution in 0.5 M NaCl, different substances (0.15 M final concentration) were added, and their effect on Δ was compared in the microscope cell with controls with and without 0.03 M ATP. After the examination 2 ml. of the various solutions were pipetted into 0.25 ml. ATP solution (0.03 M final concentration) and the Δ was measured again immediately after mixing. Controls were set up to measure the fall of Δ due to dilution. As shown in Table XVI inorganic orthophosphate, pyrophosphate, metaphosphate, hexametaphosphate, diphenylpyrophosphate, adenylic acid, and inosinic acid, have no appreciable effect on the fall of flow-birefringence brought about by ATP. Inorganic triphosphate and ADP however do show an inhibition of the ATP fall of Δ in thrice precipitated myosin.

Table XVI requires a few words of explanation. The values in the first column will be seen to be all a little lower than that of the control without any addition—this is almost certainly due to a salt-like effect of the high concentration of the substances added. We have already seen that at similar molarity to the ATP they have no effect, but in this experiment it was necessary

TABLE XVI

Competitive Inhibition of the Flow-Birefringence Reduction by Sodium Triphosphate and Adenosinediphosphate

Thrice precipitated myosin; ATP at 0.03 M; all other substances at 0.15 M.

Experiment No.	Protein concentration	Substances	Δ° at 514 R.P.M.			Per cent of best possible fall
			Δ_i	Δ_{atp}	Fall of Δ	
app/352	3.58	Control; no addition	64	58	27	100
		Control; ATP	37	31		
		Orthophosphate	53	33	25	93
		Pyrophosphate	52	30	28	104
		Metaphosphate	64	33	25	93
		Hexametaphosphate	65	30	28	104
		Triphosphate	50	37	21	77
		Phenylpyrophosphate	48	28	30	111
		Adenylic acid	58	30	28	104
app/355		Inosinic acid	58	30	28	104
		Control; no addition	76	67	20	100
		Control; ATP	55	47		
		Adenosinediphosphate	80	57	10	50
		Triphosphate	78	61	6	30
		Orthophosphate	66	46	21	103
		Pyrophosphate	72	45	22	107
		Phenylpyrophosphate	71	46	21	103

to raise their concentration in order to demonstrate any inhibition. It will also be noticed that the values for the controls in the second column are less than those in the first; this is because of the extra dilution involved in adding ATP or its dilution control.

Since triphosphate is able to block the fall in the flow-birefringence of myosin brought about by ATP, it was of interest to ascertain whether it would also inhibit the phosphorolysis of ATP. The formation of inorganic P from ATP does in fact appear to be inhibited by the presence of triphosphate, which is

also split itself (see Table XVII). In each case the values obtained from samples taken from the tube containing both sodium triphosphate and ATP were significantly lower than the sum of the controls (*e. g.* 0.956 instead of 1.58 mg. P).

All these results seem to be further evidence in favour of the view that the observable changes of the optical properties of myosin solutions in the presence of ATP are connected with the enzymatic properties of myosin as adenosinetriphosphatase. Myosin splits off inorganic P from adenosinetriphosphate and inosinetriphosphate (Kleinzeller, 1942), acting on the triphosphate grouping:

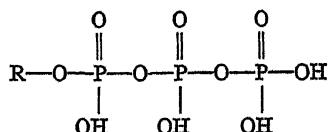


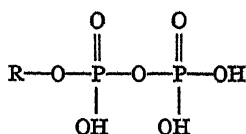
TABLE XVII

Competitive Inhibition of Adenosinetriphosphatase by Sodium Triphosphate

Final concentrations: myosin 1.48 per cent protein, 0.5 M NaCl; 0.009 CaCl₂. Final volume 2.5 ml. Incubated at 37°.

Time	Inorganic phosphorus				
	Substrate added				
	0.01 M ATP	0.015 M Na ₂ P ₂ O ₇	0.01 M ATP + 0.015 M Na ₂ P ₂ O ₇	0.01 M ATP + 0.03 M Na ₂ P ₂ O ₇	0.01 M ATP + 0.06 M Na ₂ P ₂ O ₇
<i>min.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
2	0.630	Trace	0.53	0.315	0.338
15	0.755	0.506	0.855	0.775	0.653
30	0.843	0.742	1.057	0.956	0.935

(where R can be adenosine or inosine); but does not attack the pyrophosphate grouping:



Since inorganic triphosphate has no effect on the flow-birefringence, it is concluded that both the triphosphate group and the adenosine or inosine nucleoside group are necessary to bring about the optically observable changes in myosin solutions.

DISCUSSION

About ten years ago there was much debate on what were called the "physical" and the "chemical" theories of muscular contraction (*cf.* Ritchie, 1932).

In discussions at that time, it was felt by many that "topochemical" would have been a better word than "physical." The point was very clearly put by Bernal (1938). "Do the chemical reactions (in muscle)" he said, "have to do directly or indirectly with the actual contraction process? If the first is true, they are probably of a topochemical nature, that is, reactions in which one of the reagents is not a free molecule but a radical bound in a fixed position on a chain molecule. On the other hand . . . the chemical reactions may bring about contraction indirectly by building up a potential field, probably of an electrical nature, in which the chemically inert myosin molecule contracts reversibly." The new facts seem to be showing that the first alternative is the right one. And it should perhaps be emphasised that this has further implications than the very obvious phenomenon of muscle contraction itself. It is not wide of the mark to say that all morphogenesis is a problem of protein chemistry, since we can credit only the proteins with the burden of change and maintenance of cell architecture. Hence the relation between the smaller energy-rich molecules in true solution or dispersion and the enormous protein molecules which are the bricks of cell structure is one of vital importance for the embryologist no less than the physiologist.

From the data reported in the present paper, it seems fair to say that the interaction between adenosinetriphosphatase and its natural substrate brings about deep seated and optically observable effects upon the enzyme's physical state. Whether these changes are micellar in character, or whether they are analogous to the configurational changes established for the keratins, remains as yet undetermined. Could adenosinetriphosphate itself (or, in view of the results of Kleinzeller (1942), inosinetriphosphate) then be normally *in vivo* the agent of contraction? Were this so, the reception of the nervous or other stimulus would essentially allow the enzyme and substrate to come in contact. The exact location of inorganic ions at this stage may also be important, *e.g.* Ca^{++} (Bailey, 1942), K^+ (Verzar, 1942). In this connection it may be significant that Caspersson and Theorell (1941, 1942) have been able by ultraviolet microspectroscopy to localise the adenosinetriphosphate in the non-birefringent bands of the *resting* muscle fibre. By this enzyme-substrate combination, then, the configuration of the contractile enzyme would itself immediately be changed (as pictured by Astbury and Dickinson, 1940; Astbury, 1942). Further experiments upon the quantitative relationship between the ATP present and the change produced in double refraction and upon the time relationship of the optical changes and the enzymic breakdown are still needed to decide between the two possibilities: (a) That the combination between myosin and ATP so alters the myosin molecule, that reaction between some groups along the length of the chain becomes possible and provides energy. The subsequent splitting off of phosphate from the substrate would then supply energy needed for relaxation and recharging of the myosin fibril. (b) That

the splitting off of phosphate and setting free of energy from the ATP accompanies the contraction. In any case, it must be remembered that, *in vitro*, the contraction of the myosin particles does no work, and the conditions are therefore very different from those *in vivo*. This objection might be met if it should prove possible to do enzyme experiments with myosin fibrils strong enough to withstand a measurable tension without breaking. (See the experiments of Engelhardt, Ljubimova, and Meitina (1941) referred to later.)

From the facts already known arises another alternative suggestion (entertained for some time past by one of us and now put forward in a rather different form by Kalckar, 1941), that myosin itself might be phosphorylated and dephosphorylated, occupying thus the last link in the chain of simultaneous transfers of phosphate ions and energy. As is well known, adenosinediphosphate can be phosphorylated according to circumstances by creatinephosphate, phosphopyruvic acid, or diphosphoglyceric acid. One may suggest that while adenosinediphosphate acts as a phosphate acceptor towards these substances, adenosinetriphosphate acts as a phosphate donor towards the contractile protein itself. Contracted myosin would thus have adenosinetriphosphatase activity, and phosphate would be transferred from the adenosinetriphosphate to some part of the protein molecule, which would simultaneously extend. Extended myosin would then be in phosphorylated form and charged with energy. When the physical changes touched off by the nerve stimulus occur, inorganic phosphate would be set free, and the energy available from the dephosphorylation used in contraction. Experiments on the possible phosphorylation of myosin are in progress by one of us.

Very occasionally, in two myosin preparations in our experience, adenosinetriphosphate was found to exert no effect on the flow-birefringence of the protein. We can offer nothing to account for this observation, but it may be remarked that in one of these cases the myosin had been prepared from a rabbit which had been subjected to convulsions before being killed.

In considering the effect of ATP on myosin it must always be remembered that the enzyme action and the physical changes are, under certain conditions, separable. In early preparations which were not flow-birefringent, the enzyme was active. Conversely, after treatment at 37°C. the flow-birefringence is retained or enhanced but the enzyme action is largely destroyed. The first case may have several explanations (a) the protein may be already "set" in the fully contracted state; (b) its particles may have too small an axial ratio to allow of the normally occurring changes being seen; (c) the ATP may only link on at the phosphate end and be split like inorganic triphosphate. In the second case, the protein micelles may be "set" in the fully extended state.

The possibility, therefore, must still be borne in mind that the enzymic activity (adenosinetriphosphatase) may involve some grouping separable from the contractile protein itself. In this case the optically observable effects of ATP on myosin would be the result of changes conveyed through this grouping.

If it should prove that the changes are molecular rather than micellar, then one might picture the molecular length of adenosinetriphosphate as being shorter than the length between the groups on the myosin chain to which it becomes attached. Contraction would then necessarily accompany combination. This would be in line with the general two-affinity theory of enzyme action, only in globular corpuscular proteins configurational changes in the enzyme protein would not be so readily observable as in linear corpuscular (soluble fibrous) proteins.

The evidence for the identity of myosin and adenosinetriphosphatase, resting upon the failure of all attempts to separate them, must remain of a negative character. But the fact that far reaching optically observable changes in the myosin accompany the enzyme-substrate combination and the subsequent enzyme action, gives positive support to the view that the enzyme is myosin itself. It may therefore now be allowable to speak for the first time of a "contractile enzyme." And the present work provides suggestive evidence that the enzyme-protein contracts "in solution."

One cannot, of course, compare too closely the behaviour of myosin in the muscle cell and myosin isolated from it in the form of a sol. In the muscle the contracting particles do work against resistance; in the sol this does not happen. In the muscle, only a very small proportion of the myosin can be in true sol form, such as we see it *in vitro* (Bate-Smith, 1934). It is interesting, however, to compare the birefringence effects seen on isolated purified myosin *in vitro* with those which may be observed in the intact muscle fibre. It is known from older work, put on a firm basis by von Muralt (1932) that during a single isometric twitch the birefringence of the intact muscle fibre decreases by some 35 per cent, returning to its original value by the end of relaxation. It has also been found by Buchtal and Knappeis (1938) that in the absence of contraction, changes in pH and salt concentration in the medium surrounding a contractile isolated muscle fibre, bring about slower reversible changes in its birefringence. We have seen, too, in the first part of this paper, that many chemical agents exert effects on the flow-birefringence of myosin similar to those which they exert on the birefringence of the intact muscle fibre.

From the important work of Stübel (1923) we know that this birefringence of the intact muscle is due to three components (*a*) a positive rod form-birefringence, (*b*) a positive intrinsic birefringence, and (*c*) a negative intrinsic birefringence. The first two are certainly due to the protein, the third to associated lipins. Already in 1923 Stübel could confidently write "In den Muskelfaser finden sich gleichsinnig orientierte, stäbchenformige, kristallinische, doppelbrechende, Eiweissteilchen." Later, Stübel and Liang (1928) showed that the birefringence of intact muscle decreases and is lost in rigor or contractures, whether isotonic or isometric.

The "viscosity" or viscous elasticity of the intact muscle has also been the subject of important investigations (Hill, 1922; Gasser and Hill, 1924; Levin and Wyman, 1927; Stevens and Metcalf, 1934; Fenn and Marsh, 1935). They seem to show that the viscosity of a muscle on tetanus or even during a single twitch is at least ten times that of the same muscle at rest. But it is impossible to compare these data on the

highly packed myosin gel within the cell membrane with ours on the freely orientable particles of the myosin sol. If indeed the intracellular pH shifts slightly towards the acid side, the *in vivo* phenomenon might be connected with the high viscosity seen on the left-hand side of Fig. 5 in the present paper.

We doubt if there is anything to be gained by referring to the myosin sol after its flow-birefringence has fallen and it has begun to dephosphorylate adenosinetriphosphate as "denatured." Such a "denaturation" (which involves neither changes of solubility, nor as has been shown by Greenstein and Edsall (1940) in the case of the salt effects, changes in titratable —SH linkages) has not gone beyond an extremely early reversible stage at which it is compatible with active enzymic breakdown of the adenosinetriphosphate. As we have noted in Paper II of this series (1944) enzymic activity may continue actively even after an enzyme-protein has undergone denaturation by uncoiling in the surface film at an air-water interface. But the evidence we bring forward here shows that the relative viscosity of myosin falls under the action of ATP, whereas in denaturation it should rise; and the flow anomaly is retained, whereas it should be lost.

A case which is perhaps relevant to the present one, though not closely analogous to it, is the fall in flow-birefringence and anomalous viscosity of polymerised sodium thymonucleate brought about by proteins and the "depolymerase" (Greenstein and Jenrette, 1941, 1942; Greenstein, 1942) and by ultraviolet irradiation (Hollaender, Greenstein, and Jenrette, 1941). These effects, however, are not reversible. It does not seem as yet clear that they are due to disaggregation (depolymerisation) as against deformation or tangling of the micelles, though admittedly we know of other, purely chemical, evidence for "ribonucleo-depolymerase" (Schmidt and Levene, 1938).

If, as we assume, the change in flow-birefringence (and hence in particle shape) of myosin in presence of adenosinetriphosphate is a manifestation of the enzyme-substrate combination, it would take its place among the few other such directly observable processes, such as the spectral shifts of catalase (Keilin and Hartree, 1937) and peroxidase (Keilin and Mann, 1937) and the visible colour changes of certain flavoproteins (Haas, 1937). It would be, however, the first example of such an effect observable by polarised light. Little else is known about the combination of myosin and adenosinetriphosphate, save that no change in the ultraviolet absorption spectrum of the protein is to be seen; the curves of the two substances together are summative (Ljubimova and Shipalov, 1940). A non-summative change, however, would not necessarily be expected, since ultraviolet absorption primarily indicates ring structures, and no changes in these are likely to happen when myosin and adenosinetriphosphate combine.

From the data reported above, the conclusion is probably justified that myosin is a specific triphosphatase. Since inorganic triphosphate combines with

the protein and is split without causing any fall of Δ , we may say with some plausibility that the purine-ribose end of the nucleoside-triphosphate molecule is also of much importance in changing the shape of the protein molecule or micelle.

An interesting analogous case to this has recently been published by Buchman, Heegaard, and Bonner (1940). Thiazol-diphosphate (thiazol-pyrophosphate), which has a similar constitution to aneurin (thiamin) diphosphate, save that it lacks the methyl-amino-pyrimidine ring, inhibits the activity of carboxylase, competing with thiamin-diphosphate itself, if the latter is present. Here a coenzyme, cocarboxylase, therefore, which is thought normally to become attached to the enzyme-protein by two bonds (Stern and Melnick, 1939), is kept away from the protein, and hence the activity of the whole system destroyed, by a closely related substance which becomes attached by only one bond and has no coenzymic activity. In the case of myosin, of course, the smaller molecule is substrate, not coenzyme; but there is an analogy in the blockage of optical effect and phosphorolysis by inorganic triphosphate.

The only other attempt which has been made to elucidate the relationships of adenosinetriphosphate and myosin, which today seem crucial for any understanding of protein contractility, is that of Engelhardt, Ljubimova, and Meitina (1941). Partially dried fibres of myosin, prepared by injection from extruders into distilled water, and still retaining some adenosinetriphosphatase activity, were treated with a variety of substances, of which alone adenosinetriphosphate showed any effect—an increase of extensibility. The concentration of adenosinetriphosphate was similar to that found by us to be minimal for complete effectivity, that is, 0.0025 M. It is, of course, impossible to compare readily the results of two techniques so different, especially as the full details of the Russian work are not yet available. Much may be expected from the x-ray analysis of such fibres parallel with determinations of enzyme activity, if this should be technically possible. The effects of electrolytes on partially dried myosin fibres have recently been examined by Gerendas and Szent-Györgyi (1941), but the paper has so far been available to us only in the form of an abstract.

The observations and experiments discussed in this paper may at first sight seem to have little bearing on the problems of interest to the embryologist with which the first and second papers of the series partly dealt. But the significance of protein contractility is far wider than the muscle problem alone. The contractility of intra- and intercellular fibrils in various embryonic processes has often been pointed out (*cf.* J. Needham, 1942). The problem of energy transfer and change in configuration of protein fibrils is not without relevance to inductor-reactor systems in embryos undergoing morphogenesis. The possibility should now be borne in mind that an inductor substance might be, not a hormone, nor a coenzyme nor an autocatalytically active protein,

but a substrate, and that the changes in protein configuration which will lead to a specific path of histogenesis and all that that implies, might be the result of the unavoidable action of an enzyme-protein in the competent cell upon the inductor itself.

SUMMARY

1. An investigation of the physicochemical properties of myosin has been carried out. Prepared under standard conditions, the ratio of flow-birefringence to protein concentration is uniform. The effect of electrolytes, pH, and urea on the flow-birefringence and viscosity (relative and anomalous) of myosin has been examined.

2. Decrease or abolition of flow-birefringence does not necessarily imply far reaching denaturation, since such effects can be reversed by a variety of means.

3. When a myosin solution is treated with adenosinetriphosphate, its flow-birefringence is decreased (average 48 per cent), its anomalous viscosity is retained, and its relative viscosity is decreased (average 14 per cent). The full effect of adenosinetriphosphate is obtained at 0.004 M; a molarity very much less than that of other substances which decrease the flow-birefringence of myosin.

4. The changes in the physicochemical properties of myosin brought about by adenosinetriphosphate are spontaneously reversible, and are connected with the enzymatic action of the protein as adenosinetriphosphatase.

5. Effects similar to those of adenosinetriphosphate on the physicochemical properties of purified myosin have been obtained so far only with inosinetriphosphate.

6. Inorganic phosphate is split off by myosin from inosinetriphosphate as well as from adenosinetriphosphate. Inorganic triphosphate is split by 1 to 2 per cent solution of three times precipitated myosin.

7. Adenosinediphosphate and inorganic triphosphate act as competitive inhibitors with adenosinetriphosphate, blocking the fall of flow-birefringence.

8. The implications of the results, and the conception of active enzymic groups attached to proteins participating in cell structure, whether contractile or non-contractile, are discussed in relation to present views on muscle physiology and other biological problems.

The authors are glad to have this opportunity of recording the perennial inspiration of Sir F. G. Hopkins, O.M., whose own work has been so central in the history of muscle physiology. They also wish to thank Professor C. E. Tilley and Dr. C. Phillips for the loan of apparatus used in this investigation. For the specimens of tobacco mosaic virus used they would thank Mr. R. Markham; for the gift of supplies of lithium chloride, Kemball, Bishop and Co.;

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THEORY AND MEASUREMENT OF VISUAL MECHANISMS

XI. ON FLICKER WITH SUBDIVIDED FIELDS

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I

When visual flicker is produced by passing vertical dark bars across an illuminated field¹ with fixed inclined opaque stripes on it, certain pronounced changes are induced in the properties of the flicker contour.^{2, 3}

Ordinarily, with image fields not subdivided, and regardless of whether flicker is produced by sectoring the light beam at a focus⁴ or by the striped cylinder technic, the dependence of the contour for flicker recognition on the light-time fraction t_L in the flash cycle is direct and simple.⁵ The maximum flash frequency F to which the $F - \log I$ curve asymptotically rises declines rectilinearly with increase of t_L ; its abscissa of inflection (photopic "cone" segment) increases rectilinearly with t_L ; and the third parameter of the probability summation⁶ describing the simplex $F - \log I$ curve, namely the standard deviation $\sigma'_{\log I}$ of its first derivative in $\log I$, with $F_{max. put} = 100$, is found not to change at all when t_L is altered. (In certain cases, with most arthropods,⁷ slight changes in the shape of the lower part of the curve arise as a consequence of the convexity of the surface of the eye, but this does not affect the essential generality of the previous statement.) Fundamentally, the behavior of the scotopic "rod" part of duplex $F - \log I$ contours is the same, but this may be obscured by the integrated overlapping of the "rod" and "cone" populations of neural effects.⁸

With an obliquely barred field, however, and flicker produced by evenly

¹ Wolf, E., and Zerrahn-Wolf, G., 1935-36, *J. Gen. Physiol.*, **19**, 495. Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936-37, *J. Gen. Physiol.*, **20**, 211; 1937-38, **21**, 203, etc.

² 1941-42, *J. Gen. Physiol.*, **25**, 369.

³ 1943-44, *J. Gen. Physiol.*, **27**, 287.

⁴ 1940-41, *J. Gen. Physiol.*, **24**, 505, 635; 1941-42, **25**, 89, 293; 1943-44, **27**, 119.

⁵ 1937-38, *J. Gen. Physiol.*, **21**, 313, 463; *Proc. Nat. Acad. Sc.*, 1940, **26**, 60; *J. Gen. Physiol.*, 1939-40, **23**, 531.

⁶ 1938-39, *J. Gen. Physiol.*, **22**, 311, 451; 1940-41, **24**, 625; 1941-42, **25**, 369; 1943-44, **27**, 119.

⁷ 1937-38, *J. Gen. Physiol.*, **21**, 463.

⁸ 1937-38, *J. Gen. Physiol.*, **21**, 313; 1940-41, **24**, 635.

spaced vertical bars moving across it, the shape constant $\sigma'_{\log I}$ is sharply decreased above $t_L = 0.50$, so that the contour is then much steeper; τ' does not increase as much as with a plain field, and neither τ' nor $F_{max.}$ is a simple function of t_L . These effects cause the curves for large values of t_L to cut across those for the smaller values, instead of being evenly and symmetrically spaced as with the plain field.

This latter picture is the one obtained for the sets of $F - \log I$ contours at different values of t_L secured with birds,^{3,9} by the revolving stripe method. The birds used possess large, well developed pectens. The theory³ is that the moving contact of bar images with the serrated shadow of the pecten causes the natural occurrence of the phenomenon experimentally induced in man by the use of the obliquely barred field.

Partly as a test of this conception, partly for other reasons, we have examined the results to be obtained when transilluminated parts of stationary barred patterns of different sorts are caused to flicker simultaneously by sectoring the light at a focus. The point then is that, a standing pattern being intermittently illuminated, one can inquire whether subdivision of the flickered field into several parts will of itself introduce modification of the properties of the set of flicker contours when t_L is varied, and whether such modification will be similar to that involved in the "pecten effect." From another standpoint, it is to be noted that we can also examine by this means the problem of neural integration in visual excitation.¹⁰ The relation between F and $\log I$ critical for flicker, although simultaneously apparent in the several parts of a subdivided field, is not the same as when one of its parts is tested separately or when a simple field of the same general form and the same total illuminated area is flickered.

We are more immediately concerned with the demonstration that the "pecten effect" is only in part reproduced in the changes actually effected in the properties of the $F - \log I$ contour by the "stationary flickering" of subdivided fields. The character of the changes found substantiates the importance of the "sliding contact" of stationary and moving dark images for the production of enhanced flicker acuity by the "pecten effect."

The results here given also extend the basis for an understanding of the manner in which "rod" and "cone" effects are integrated to produce the $F - \log I$ contour in the region of their overlapping. They likewise bear directly upon the curious properties of "visual acuity" with interrupted light, which we discuss in a following paper. And, in a more general connection, the data here cited complete the proof that three independently modifiable parameters ($F_{max.}$, τ' , $\sigma'_{\log I}$) are involved in the nature of the flicker contour.

⁹ 1943-44, *J. Gen. Physiol.*, **27**, 315.

¹⁰ Cf. 1940-41, *J. Gen. Physiol.*, **24**, 505; 1941-42, **25**, 369; and a following paper.

II

The general procedure followed in these experiments, the instrumentation, and the methods of calculation, have been described previously.¹¹ The four fields used for the present discussion are: (i) a square subtending at the retina 3° on a side, centered 6° horizontally on the temporal aspect of the left retina; (ii) a square, subtending 3° on a side, with horizontal and vertical subdivision into four equal squares (Fig. 1), the opaque dividing stripes being 0.3° broad; centered as for (i). The crossed bars were formed of pieces of wristwatch hair-spring mounted in the jaws of the spectroscopic slit in one arm of our discriminometer.¹² The total illuminated area with (i) and (ii) was taken to be nearly enough the same, despite scattered light on the crossbars. (iii) A square subtending 10° on a side, centered at the fovea, with three opaque vertical bars each 1.43° wide, thus giving four vertical illuminated spaces of that width, equally separated; and (iv) a square 10° (really 9.91°) on a side with six opaque vertical bars and seven illuminated bars all of 0.77° width. Thus the bars in (iv) were of about one-half the width of those in (iii), and the total illuminated area in (iv) was a little less (52.9 square degrees as compared with 57.2 square degrees). For certain tests field (iv) was rotated 90° so that the stripes were horizontal; we may speak of this as field (v). The opaque bars were produced by hard photographic reduction of carefully made contact prints of Levy plates, mounted in the spectroscopic slits of the discriminometer.

The observations were made monocularly (left eye). Tungsten white light was used with (ii), and the white and a red and a blue filtered from it with the other fields.

III

The data for fields (i) and (ii) are given in Tables I and II. In Figs. 1 and 2 it is seen that the nature of the shift in contours with change of light-time fraction t_L is of the kind already found for plain fields.¹¹ In the subdivided field (ii) there is no change of the slope constant at $t_L = 0.90$, nor any change in the type of relation of τ' to t_L ; and in this sense there is no pronounced "pecten effect."^{3,9} But the picture is nevertheless changed considerably when the 3° square is subdivided into four parts. The "rod" segment is then greatly enlarged, the "cone" segment becomes much steeper, and its midpoint is moved to a higher flash intensity, the interrelation of these two phenomena we shall consider shortly. The slight increase in over-all illuminated area in (ii), by scattered light on the crossbar images, cannot possibly account for them.¹³ The steepening of the "cone" segment is a prominent feature of the "pecten effect," but when this effect occurs it is seen only with longer light-times in the flash cycle;^{3,9} here it is independent of t_L . It is perfectly clear, as already emphasized elsewhere,¹⁰ that pronounced changes of the shape constants of the flicker contours make futile any attempt to deduce from such constants alone

¹¹ 1940-41, *J. Gen. Physiol.*, **24**, 505, 635; 1941-42, **25**, 89, 293.

¹² 1938-39, *J. Gen. Physiol.*, **22**, 341.

¹³ Cf. following paper.

TABLE I

Data for flicker recognition contours with a 3° square image centered 6° on the temporal side of the fovea (W. J. C., monocular observations with *left* eye); white light; flash intensities I in millilamberts; " t_L " = $t_L/(t_L + t_D)$; $n = 10$ for each point.

F per sec.	$t_L = 0.10$ $\log I_m$ log P.E. ₁	$t_L = 0.50$ $\log I_m$ log P.E. ₁	$t_L = 0.90$ $\log I_m$ log P.E. ₁
2		$\bar{6}.6095$ $\bar{7}.0405$	$\bar{5}.0686$ $\bar{7}.5487$
4		$\bar{6}.6736$ $\bar{7}.1897$	$\bar{5}.0723$ $\bar{7}.5454$
5		$\bar{6}.9373$ $\bar{7}.7479$	$\bar{5}.4216$ $\bar{7}.8757$
7		$\bar{6}.9346$ $\bar{7}.3341$	$\bar{5}.5516$ $\bar{6}.1567$
8	$\bar{6}.3649$ $\bar{8}.8924$	$\bar{5}.2384$ $\bar{6}.5384$	$\bar{5}.5456$ $\bar{6}.0957$
9			$\bar{5}.8187$ $\bar{6}.4270$
10	$\bar{6}.6949$ $\bar{7}.1411$	$\bar{5}.5753$ $\bar{6}.1377$	$\bar{5}.9594$ $\bar{6}.4233$
12	$\bar{5}.0962$ $\bar{7}.5690$	$\bar{5}.9848$ $\bar{6}.4544$	$\bar{5}.9996$ $\bar{6}.3734$
15	$\bar{5}.7021$ $\bar{6}.1747$	$\bar{4}.5838$ $\bar{5}.0917$	$\bar{4}.2148$ $\bar{6}.6676$
18	$\bar{4}.4195$ $\bar{6}.9416$	$\bar{3}.2765$ $\bar{5}.7621$	$\bar{4}.4717$ $\bar{5}.1499$
20	$\bar{4}.7667$ $\bar{5}.2744$	$\bar{3}.6166$ $\bar{5}.9389$	$\bar{4}.4758$ $\bar{6}.8742$
22	$\bar{4}.9610$ $\bar{5}.3297$	$\bar{3}.8228$ $\bar{4}.2854$	$\bar{4}.9366$ $\bar{5}.5446$
25	$\bar{3}.2548$ $\bar{5}.8353$	$\bar{2}.1268$ $\bar{4}.5204$	$\bar{4}.9349$ $\bar{5}.4200$
30	$\bar{3}.7843$ $\bar{4}.2037$	$\bar{2}.6770$ $\bar{3}.1989$	$\bar{3}.4493$ $\bar{4}.1306$
33	$\bar{3}.7913$ $\bar{4}.2042$		$\bar{3}.4767$ $\bar{4}.0262$
35	$\bar{2}.2567$ $\bar{4}.7288$	$\bar{2}.9478$ $\bar{3}.3516$	$\bar{2}.1467$ $\bar{4}.6338$
38		$\bar{1}.0934$ $\bar{3}.6108$	$\bar{2}.1235$ $\bar{4}.5924$
40	$\bar{2}.8010$ $\bar{3}.4135$	$\bar{1}.4495$ $\bar{2}.0345$	$\bar{2}.4818$ $\bar{4}.9490$
43		$\bar{1}.6660$ $\bar{2}.1924$	$\bar{2}.4929$ $\bar{4}.9477$
45	$\bar{1}.5326$ $\bar{3}.9451$	0.0697 $\bar{2}.5342$	$\bar{2}.4729$ $\bar{4}.9179$
47		0.4681 $\bar{2}.8685$	$\bar{2}.7069$ $\bar{3}.2570$
48	0.2858 $\bar{2}.6592$	0.6931 $\bar{1}.1354$	$\bar{2}.7207$ $\bar{3}.2527$
49		1.2014 $\bar{1}.7156$	$\bar{2}.9932$ $\bar{3}.4692$
50			$\bar{1}.0022$ $\bar{3}.4720$
51	1.5486 $\bar{1}.8261$	2.3454 0.8464	$\bar{1}.3801$ $\bar{3}.8726$
52		2.3420 0.9756	$\bar{1}.3860$ $\bar{3}.8497$
		3.4087 1.0384	$\bar{1}.7179$ $\bar{2}.2809$
			$\bar{1}.8988$ $\bar{2}.2872$
			$\bar{1}.9057$ $\bar{2}.3942$
			0.2808 $\bar{2}.8894$
			0.5603 $\bar{1}.0728$
			0.5470 $\bar{2}.9529$
			0.9619 $\bar{1}.5318$
			1.3294 $\bar{1}.9665$
			1.3440 $\bar{1}.8679$
			1.8013 0.2591
			2.1970 0.9468
			2.2201 0.6583
			3.0287 1.4591
			2.9605 1.4342
			2.9843 1.4817

clues as to the physicochemical character of the primary excitation process. Mere subdivision of the illuminated area could not possibly change "reaction

TABLE II

Data for flicker contours obtained with a square image area totaling 9 square degrees, subdivided into four equal squares by vertical and horizontal cross-bars each 0.3° wide; the center of the cross being 6° on the temporal side of the fovea. These data are to be compared with those in Table I for the simple $3^\circ \times 3^\circ$ square all in one piece. Observer W. J. C., left eye; $n = 10$.

F per sec.	$t_L = 0.10$		0.50		0.90	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
2	5.5296	6.0437	4.0358	6.5216	4.5362	5.0705
6	5.8706	6.1621	4.3535	6.7251	4.8953	5.3624
10	4.1544	6.5098	4.6482	6.9960	3.1878	5.6541
14	4.5731	5.1187	3.0715	5.5204	3.5393	5.9576
16					3.8075	4.2777
17	4.9337	5.5105				
18			3.6185	4.0338	2.1664	4.4564
19	3.4863	5.9983	2.0496	4.4241	2.5256	4.9719
20	3.7858	4.1610	2.5606	4.9877	1.3895	3.7798
	3.7932	4.1414	2.5841	4.9999	1.4338	2.0034
22	2.4901	4.9249	1.0633	3.5854	1.6647	2.1363
	2.4984	4.8552	1.1018	3.5762	1.6244	2.0608
25	2.9091	3.4289	1.4815	3.9960	1.8952	2.3080
			1.3226	3.7931		
			1.4112	3.7547		
30	1.2251	3.6523	1.8242	2.1775	0.1718	2.6365
			1.6628	2.0844		
			1.6813	2.0173		
35	1.5678	3.9732	1.9635	2.1273	0.4717	2.8669
			0.0146	2.5026		
40	1.8965	2.3267	0.3827	2.7324	0.8227	1.2419
			0.3683	2.7843		
45	0.4712	2.8844	1.0082	1.6089	1.4786	1.8508
			0.9528	1.3601		
48	0.9999	1.5367	1.3675	1.6347	2.0461	0.4123
			1.5210	1.8894		
			1.5217	0.0420		
50	1.5181	1.9547	2.1603	0.7309	2.7521	1.3171
			1.9760	0.4015		
51	2.6747	1.1632	2.6747	1.1632		
			3.0448	1.5308		

orders." Table II includes at $t_L = 0.90$ two sets of measurements made about a year apart. They show good agreement.

In correlation with the abrupt steepness of the photopic curves in Fig. 2, we note that F_{max} does not change so rapidly with t_L (Fig. 3 *b*), although the rate

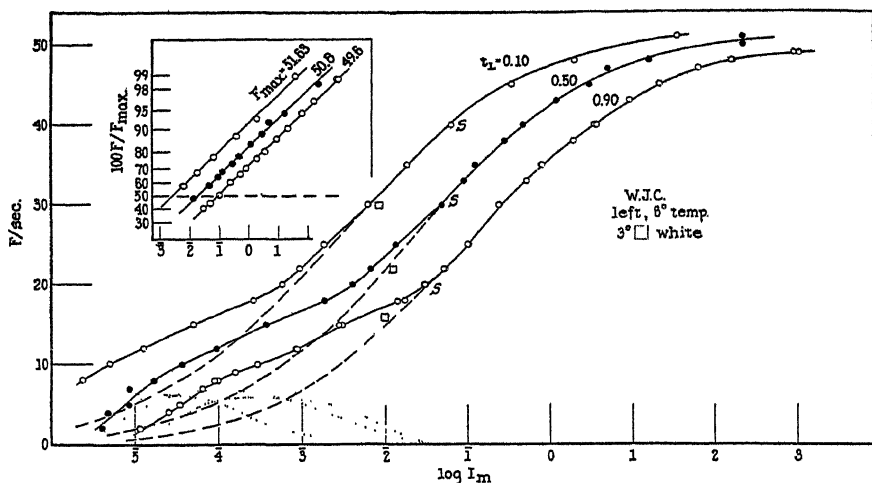


FIG. 1. $F - \log I$ contours for a 3° square image, centered 6° horizontally on the temporal side of the left fovea; white light; light-time fractions $t_L = 0.10, 0.50, 0.90$. The inset figure shows the upper portions of these curves on a probability grid, computed to the values of F_{max} indicated; to show that the slope constants ($\sigma'_{\log I}$) are identical. These probability integrals are shown extrapolated, below, and the separated "rod" contributions are given by the dotted lines. Data in Table I.

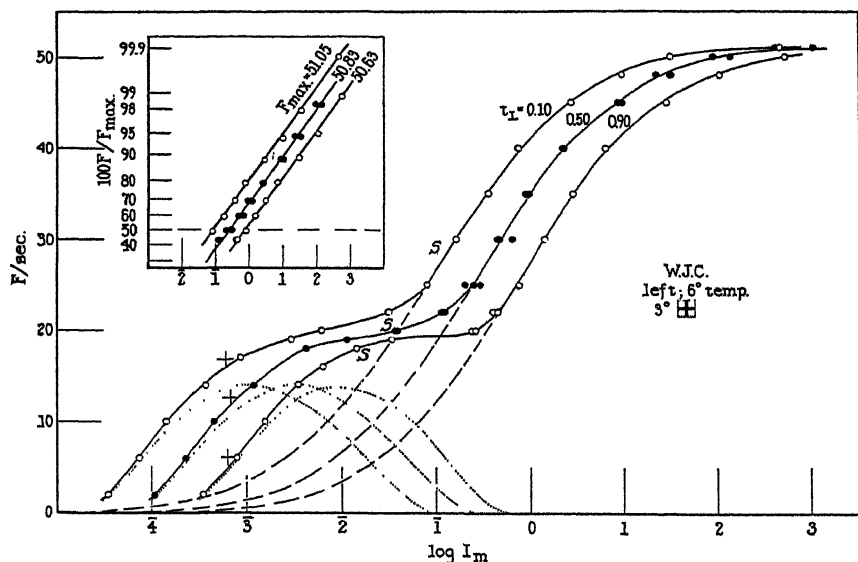


FIG. 2. Conditions as in Fig. 1, but here the 3° square has been subdivided into four equal parts, separated by dark bars 0.3° broad (see text). Data in Table II. In comparison to the curves in Fig. 1, $\sigma'_{\log I}$ is less, but is again the same for the three values of t_L . The scotopic segments are much larger than in Fig. 1. The ascending and the descending branches of the separated-out "rod" components are probability integrals drawn with $F_{max} = 14.25, 14.25, 13.9$.

of change of the abscissa of inflection τ' (Fig. 3 *a*) is about the same as for the undivided field of the same total illuminated area.

In general, above a certain small size, increase of image area A in a given retinal region causes the "cone" curve to become steeper.¹³ Comparisons with contours for image sizes larger and smaller than $3^\circ \times 3^\circ$, centered at 6° from the fovea, show that the slope increases with A in this way.¹³ The interpretation that this is due to the involvement of a larger number of cone units is consistent with the facts that the slope constant $\sigma'_{\log I}$ is (*a*) independent of t_L and of temperature,¹⁴ although a function of wave length; and (*b*) it changes in the expected way when a given illuminated patch is placed at different locations on the retina where the numbers of cone units differ. The evidence is consid-

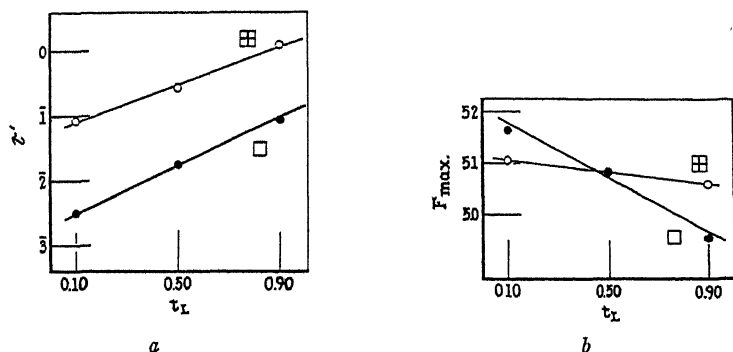


FIG. 3. The rectilinear relations of τ' to t_L and of $F_{\max.}$ to t_L , are shown for the (photopic) $F - \log I$ contours of Figs. 1 and 2.

ered in some detail in a following paper.¹³ The argument for the necessity of dealing with all the available units at all levels of F and I has been set out previously.¹⁵

The question arises whether this can be the explanation for the difference between the "cone" slope constants in Figs. 1 and 2. We believe that it can. The flicker end-point is conceived to be brought about, neurally, by the production of a certain frequency of what we have termed "elements of effect;" the same end-point, namely recognition of flicker, can be achieved by a smaller average number of such elements from each of a larger number of neural units or by a greater mean contribution from each of a smaller number of units. Subjectively, at the end-point, all of the illuminated field, subdivided or not, is seen to flicker at once, but the effect is sharper at the boundaries. It is important to realize that the modification of the flicker contours with field (ii)

¹⁴ 1937-38, *J. Gen. Physiol.*, **21**, 313; 1938-39, **22**, 311; 1939-40, **23**, 531; 1940-41, **24**, 635; 1941-42, **25**, 89, 293, 369; 1943-44, **27**, 119.

¹⁵ 1943-44, *J. Gen. Physiol.*, **27**, 119.

appears well below the brightness level at which there is perception of the fact that the field is actually subdivided (*e.g.*, with $t_L = 0.10$, the bars are not visible until a flash intensity of $4.80 \log \text{ ml. units}$ is reached). In comparison to the 3° square field, the square broken into four parts has twice the perimeter of light/dark separation. The $F - \log I$ contours can be compared with those for a 6° square, having very nearly the same perimeter as that of field (ii). The comparison is not altogether simple, since the shifts of τ' with t_L are not the

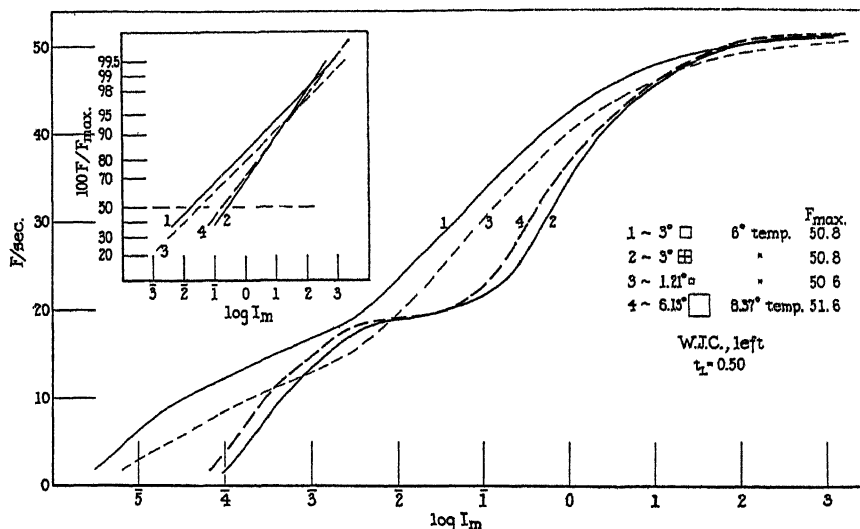


FIG. 4. Certain $F - \log I$ contours, $t_L = 0.50$, for square images (white light), centered at *ca.* 6° on the temporal side of the left fovea (W. J. C.), are traced for comparison. See text. The heavier continuous line for the 3° square area subdivided into four equal parts approaches quite near to the heavier dashed line for the simple 6.13° square (centered 8.4° off the fovea; there is no real difference produced, for such a square, by the slightly more temporal centering, which, however, has for our purpose certain advantages).

The inset graphs show the upper parts of the main contours on a probability grid. See text.

same, but qualitatively the indications are suggestive (Fig. 4). Here we have traced the curves for several image areas at $t_L = 0.50$. The contours for the 6° and the subdivided 3° squares are even closer together at $t_L = 0.90$; $t_L = 0.50$ was chosen because there the several values of $F_{\max.}$ are closest together. Fig. 4 shows that one of the four parts of field (ii) gives a curve very little changed from that for the simple 3° square (i), but that doubling the extent of the light/dark edge, keeping A constant, has about the same effect as doubling the size of the square. The conception of a smaller mean contribution with (ii)

from each excitable unit, that is a smaller frequency of elements of effect from each, is strengthened by the greatly reduced change of F_{max} with change of t_L .

This general conclusion as to the significance of the lines of light-dark separation is reinforced by the findings in the experiments of section V. It has already been stressed in another connection, namely in the data on the excitation of the eyes of bees;¹⁶ but there it is primarily a matter of the total frequency with which impulses are being generated, and the eye area illuminated at any instant is not the same when more stripes are introduced into a field. On the other hand, when checker-board fields are used, with I the same, fields with the same black-white perimeter have the same excitatory value for the bee although A is quite different.

The significance of contours in the flickered field, of course, introduces a somewhat novel factor into the interpretation of tests made in search of evidence for "summation" and the like, by placing one flickered patch near to another one. The most important feature of the situation may then be, not that the total illuminated area has been increased, but that there is an unilluminated zone between the patches. This is not to say that the image area is not a significant factor, however. The interesting points arising in relation to the rôle of the form of the image, and of the differential illumination of its several parts, we cannot now discuss. One approach to the question of the relative rôles played by total illuminated area on the one hand and on the other by image contour, is undertaken in section V.

The dynamical interrelations of the groups of "rod" and "cone" effects in critical flicker have been discussed¹⁰ in terms of the partial inhibition of rod units by the activation of cone units, and the statistical summation in terms of probability integrals of the remainder with the cone effects. It follows from this conception that if the cone curve can be made steep enough, and moved to relatively higher intensities, the rising branch of the scotopic $F - \log I$ curve could be completely freed from cone involvement.¹⁰ This we have found to occur normally in certain fishes.¹⁷ The effect has been produced in the human $F - \log I$ contour by imposition of several kinds of special conditions,¹⁸ but ordinarily the "rod" contribution must be extracted by subtraction of ordinates of the extrapolated "cone" curve.¹⁹ The present data on fields (i) and (ii) illustrate and justify these principles. It is apparent (Figs. 1 and 2) that with the subdivided field the scotopic branch of the con-

¹⁶ Wolf, E., 1932-33, *J. Gen. Physiol.*, **16**, 773; Wolf, E., and Crozier, W. J., 1932-33' *J. Gen. Physiol.*, **16**, 787; Zerrahn, G. 1933, *Z. vergleich. Physiol.*, **20**, 117, 151; Wolf, E., and Zerrahn-Wolf, G., 1934-35, *J. Gen. Physiol.*, **18**, 853.

¹⁷ 1937-38, *J. Gen. Physiol.*, **21**, 17; *Proc. Nat. Acad. Sc.*, 1937, **23**, 516; *J. Gen. Physiol.*, 1938-39, **22**, 463.

¹⁸ 1941-42, *J. Gen. Physiol.*, **25**, 369; 1943-44, **27**, 287.

¹⁹ Cf. 1937-38, *J. Gen. Physiol.*, **21**, 203, 313; 1940-41, **24**, 505; 1941-42, **25**, 369.

tour is greatly enlarged, although it falls at higher intensities. The analysis¹⁹ shown indicates that when the lower tail of the "cone" curve is caused to slip out from under the "rod" curve the latter increases in ordinate size, as expected. It cannot very well be supposed that the number of anatomical retinal rod units has been increased by the mere putting of crossbars on the field, or that their intensity thresholds have been profoundly increased thereby.

There are two general routes of escape from analytical difficulties created in such connections. One may suppose that the data are of neural origin, in the retina, central to the layer of primary receptors. Or it may be presumed that the quantitative properties of the data are of central nervous origin, even with

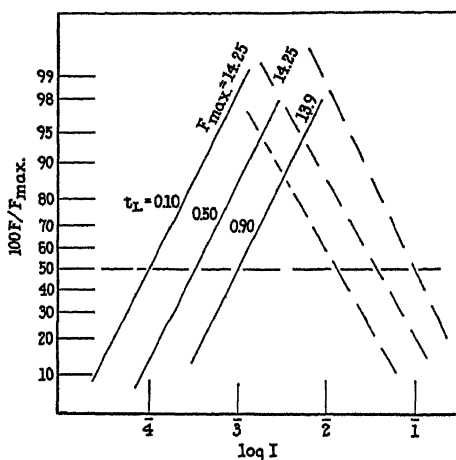


FIG. 5. The separated-out "rod" portions of the low intensity segments of the contours in Fig. 2, for the subdivided 3° square, shown on a probability grid. (The rising branches are not so steep, i.e. $\sigma'_{\log I}$ is greater, in comparison to the corresponding features for the simple 3° square in Fig. 1, signifying for the divided area an actual increase in functional number of "rod" units.)

monocular regard. There are good reasons for adopting the latter view, but they do not directly concern us now. It has been pointed out that "rod" curve flicker data, not "cone" complicated, exhibit certain significant properties which show them to be organically similar in basis of origin to the "cone" data. Thus the "rod" and "cone" curves in any one kind of animal are quantitatively shifted to the same, specific, extent as a function of change of temperature,²⁰ and of change in t_L .²¹ It is on these grounds quite impossible to assert that there is a different organic basis for the nature of "rod" effects on the one hand

²⁰ 1939, *Proc. Nat. Acad. Sc.*, **25**, 78, 171; 1938-39, *J. Gen. Physiol.*, **22**, 487; 1939-40, **23**, 143.

²¹ 1937-38, *J. Gen. Physiol.*, **21**, 313; 1940-41, **24**, 635.

and "cone" effects on the other, as exhibited in the performance contours; or to suppose that different chemical mechanisms underly them.²² This evidence is consistent with the requirements of the experimentally determined fact that the resolution of the overlapping "rod" and "cone" contributions has now been demonstrated on a simple, uniform, statistical basis, under a variety of conditions, which would not be possible if the determination of the properties of the data did not occur in a common, simultaneous locus. The results of the analysis of the variation of critical intensities are in agreement with this position.

The dissected-out scotopic functional contributions to the $F - \log I$ contours of Figs. 1 and 2 are shown on a probability grid in Fig. 5. It is clear that

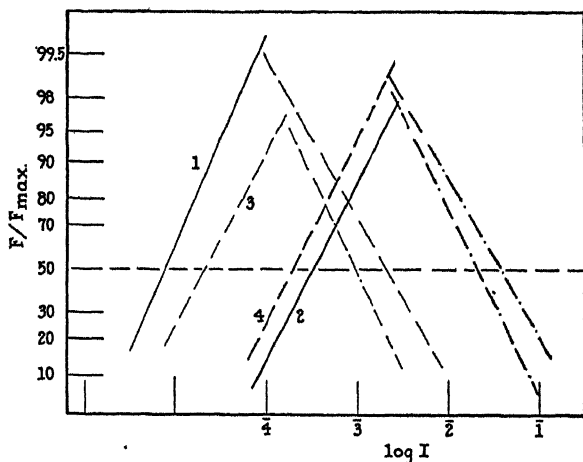


FIG. 6. Comparisons of the isolated "rod" components for the square images discussed in Fig. 4; $t_L = 0.50$.

the "rod" τ' is again a rectilinear function of t_L ; the proportionality constant is a little lower than for the "cone" branch,²³ and slightly lower than for the plain 3° square. From the comparisons in Fig. 4 it is apparent that the scotopic segment approaches that for the 6° square, and the slope of the dissected-out "rod" curve does so likewise (Fig. 6).

IV

When flicker is produced by cutting a light beam at a focus, and repeated observations are made of the intensity I_c critical for flicker, it is found in all our series of measurements that $\sigma_{1I} \pm \sigma_\sigma$ is in direct, rectilinear proportion to I_m over the whole range of F ; but that in general the proportionality factor, and

²² 1938, *Proc. Nat. Acad. Sc.*, **24**, 125; 1939, **25**, 171.

²³ Cf. 1940-41, *J. Gen. Physiol.*, **24**, 635; 1941-42, **25**, 89, 293, etc.

σ_s , are not independent of A , λ , t_L , the observer, or the eye used. All this means that the mechanism producing the data is one in which a high degree of organically determined internal correlation prevails. Part of the correlation found is due to the use of the same instrument and a reasonably fixed procedure, of course. If necessary, this factor could be experimentally identified and arithmetically extracted. But under our conditions systematic changes of the indices of variation as brought about by altering t_L , λ , or A , for example,²⁴ or the form of the image, or by using monocular *vs.* binocular regard,²⁵ must be held to reflect properties of the observer in the system giving rise to the data.

The suggestion has been made²⁶ that in "absolute" threshold measurements, and so possibly in data on discriminations at higher levels of intensity, the real source of the variability encountered is in the stimulating light delivered rather than in the observer. This idea first arose in connection with data on threshold stimulation by single brief flashes,²⁷ where the possibility, if not indeed the significant actuality, of fluctuation in the number of quanta per single flash must be taken into account. The reality of any such consideration being required when trains of flashes are used (not shorter for the single flash than *ca.* 0.009 second), with fairly large image areas, at high intensities, can be ruled out. Moreover the experimental fact is that (as we have repeatedly shown) σ_1/I_m is, for a given set of conditions, independent of intensity. But even for small, brief threshold flashes the argument proposed²⁶ is unacceptable. For this there are two reasons: the description of the dependence of responsiveness upon ΔI_0 by a Poisson summation²⁸ is not unequivocal; and if it could be shown to be unique, it could not be referred to discrete fluctuations in the "external" stimulus at the retina.

The point of the second reason is, that if integral (Poisson) variations of external light quantity occur, the mean total number of quanta being small, then in each of the media of the retina, and at each interface between media, random (Poisson) quantal losses will be suffered by the bundle of quanta in each flash, through absorption, reflection, and scattering. Now it is a fact inherent in the mathematical properties of such situations that successive superimposed Poisson effects cannot add up to produce a final Poisson distribution with respect to successive flashes. Gaussian distributions do add up in this way, and are the only ones which can.²⁸ Thus whether the frequency of positive threshold response is of Poisson form in terms of ΔI_0 , because the final number of available quanta is small, is one question; but if so it cannot be traced back to an original Poisson distribution in the initial flashes, at the retina, and consequently it cannot be said on any such basis that the essential variation in the data is due to fluctuations in the light rather than in the reacting organism.

²⁴ 1940-41, *J. Gen. Physiol.*, **24**, 635; 1941-42, **25**, 89, 293.

²⁵ 1940-41, *J. Gen. Physiol.*, **24**, 505.

²⁶ Hecht, S., Shlaer, S., and Pirenne, M. H., 1941-42, *J. Gen. Physiol.*, **25**, 819.

²⁷ Barnes, R. B., and Czernay, M., 1932, *Z. Physik*, **79**, 436.

²⁸ Cremer, H., 1937, *Random variables and probability distributions*, Cambridge Tracts, Mathematics and Mathematical Physics, No. 35, London, Cambridge University Press.

The remaining point has to do with the form of the distribution of the frequency of "successes" as a function of intensity near the threshold. The data specifically referred to in this discussion²⁶ are actually better described by a probability integral in ΔI_0 (not $\log I$), which accords with our own experience. It is specifically in agreement with the requirement of our view concerning the mode of origin of logarithmic frequency distributions in visual excitation, since for sufficiently short single flashes there is small opportunity for fluctuating performance in each excitable unit.⁶ Under these circumstances it is to be expected that nothing like the "reciprocity rule" for an inverse relation between exposure time and intensity critical for threshold should obtain, and extensive experimentation has shown us that it does not. Problems of the liminal photic energy for visual excitation have to be considered in terms of the properties of the assemblage of neural units involved.

It also accords with the meaning of results appearing when image area is varied, since then the mean liminal exciting quantity of light ($\Delta I_0 \times A \times t$) *decreases* with decrease of A down to a size so small that no exact image area can be estimated. Consequently, considerations of properties of threshold intensities in terms of energy and its fluctuations omit a factor vital to the whole situation. We therefore reject the suggestion that the fluctuations in critical intensities are not to be attributed to properties of the reacting organism.

The internal correlation manifest in the properties of σ_1 , can be estimated directly, with reasonably homogeneous data. Since the mean value of the ratio σ_1/I is independent of intensity, we can deal with the coefficients of variation collectively and write σ_1 for σ_1/I . For repeated tests with the same population sample it is known²⁹ that

$$\sigma_\sigma = \frac{\bar{\sigma}}{\sqrt{2N}} \sqrt{1-r} \quad (1)$$

where $\bar{\sigma}$ is the mean of all the S.D.'s, σ_σ is the standard error of $\bar{\sigma}$, and r is the coefficient of internal correlation. (This is, of course, usually employed for estimating σ_σ when r is known directly. Nothing in the derivation forbids reversing the argument to calculate r when σ^2 is obtained from the data.) For coefficients of variation V less than 10 per cent, as in the present case, σ_V reduces to

$$\sigma_V = \frac{\bar{V}}{\sqrt{2r}},$$

so that we can proceed with (1). Putting (1) into the form in which we deal with σ 's for the distributions of σ_1/I_m , we have

$$\sigma_{11} = \frac{\bar{\sigma}_1}{\sqrt{2}} \sqrt{1-r}. \quad (2)$$

²⁹ Cf. Peters, C. C., and van Voorhes, W. R., 1940, Statistical procedures and their mathematical bases, New York and London, McGraw-Hill.

It is this r which we can use to obtain an invariant index of "organization" or *integration* among the neural units concerned in the determination of the flicker end-point along a given contour. It is independent of the level of I of F , and thus of the level of photic adaptation. Its numerical values in the present series of experiments are quite high, but show a systematic although complex dependence upon A , t_L , λ , retinal location, and kind of image. In other types of tests of sensory discrimination its magnitude changes in a striking way. When experiments are deliberately contrived to introduce non-homogeneity into the set of data considered, the value of r drops toward zero. Thus, for a mixed set of three separated series of measurements of ΔI as a function of I_1 (W. J. C., left eye, field $12^\circ \times 12^\circ$, at the fovea, white light) we have $r = 0.101$; whereas, for a single series $r = 0.883$. In repeated independent series of flicker determinations the degree of agreement with respect to r is indicated by such findings as these: $r = 0.96, 0.97$ (3° field at 6° temporal, white light, W. J. C., left eye); $r = 0.98, 0.98$, and $0.97, 0.96$ for tests with our present field (iv). Under conditions as nearly alike as possible, r for flicker, with an undivided field, changes in a characteristic way with λ , from 0.67 (violet) to 0.97 (green).

From data previously printed we may illustrate the kind of result which r indicates. For uniocular and binocular flicker tests, with two observers, we were able to show²⁵ that for the right and left eyes respectively the mean values of σ_1/I_m were lower for the right eye than for the left, and still lower for binocular excitation. The significance of these facts for the theory of "binocular summation" has been discussed.²⁵ It is of interest to consider the values of r computed from these measurements (6.13° square, centered at the fovea, white light, $t_L = 0.50$):

	<i>L</i>	<i>R</i>	<i>B</i>
W. J. C.....	0.917	0.854	0.817
E. W.....	0.906	0.891	0.822

Thus, although the left eye ("dominant" in each case) gives a slightly higher value for σ_1/I_m than the right, and both eyes together give a value lower in the ratio of $1:\sqrt{2}$ in the average,²⁵ the "internal coherence" of the measurements, in terms of their exhibition of scatter, is slightly higher for the left and quite definitely *lower* for both eyes used simultaneously. (For auditory L, R, B measurements r behaves in a quite different way.) This result is consistent with the finding, to be set forth subsequently,¹³ that increase of flickered image area beyond a certain small size (*ca.* $1.21^\circ \times 1.21^\circ$) characteristically causes r to drop. This may be complicated by the small but definite fall of r which typically goes with increase of t_L . In general, when image area, or light-time fraction, or wave length, is increased r rises to a maximum and then declines. A given series of measurements may fall on one or another branch of this kind of curve. In view of the correlated changes in F_{max} . and in $\sigma'_{\log I}$ this is

taken to mean¹⁸ that r is a function of the number of neural units involved and also of the density of elements of sensory effect they produce.

In a considerable number of series of measurements with various simple fields, on different parts of the retina, we find (as might be expected from the indications described in earlier papers³⁰) that r is a declining function of σ_e . It has been shown that, when other things are equal, the scatter of σ_1 increases directly with the value of $F_{max.}$, and is thus a function of total number of elements of effect concerned. A test of this, which implies that in general r should rise and then decline as $F_{max.}$ increases over a sufficiently wide range, is particularly interesting in the case of subdivided fields. In our "pecten effect" experiments already described³ there occurs a decrease of $\sigma'_{\log I}$ as well as of $F_{max.}$ as t_L is increased, signifying (in terms of our analysis) that both number of units acting and mean frequency of contributed elements of effect from each unit are altered when t_L is varied. The curves for W. J. C. are pitched at lower intensities (r' is smaller), are of lower $F_{max.}$, and $F_{max.}$ and r' change more extensively when t_L is altered. This might lead to the conception that r should increase with t_L (and consequently with decline of $F_{max.}$) for the W. J. C. data, which is found: r rises steadily with fall of $F_{max.}$ for these curves, from $r = 0.68$ to 0.86 . $F_{max.}$ for E. W. at $t_L = 0.90$ is the same as for W. J. C. at $t_L = 0.10$, and the r constants there agree (0.86); but for E. W. they fall to 0.78 at $t_L = 0.10$ ($F_{max.} = 60.8$).

Applying these considerations to the measurements with our 3° subdivided square, where again σ_I and I_m are in rectilinear proportion, we find that the values of σ_e are consistently lower when the square is subdivided:

	t_L	= 0.10	0.50	0.90
$3^\circ \square$	$\log \bar{\sigma}_e$	= 3.755	3.867	3.883
$3^\circ \boxplus$	"	= 3.697	3.738	3.639

while the values of $\bar{\sigma}$ may be a little lower, but not very significantly:

	t_L	= 0.10	0.50	0.90
$3^\circ \square$	$\log \bar{\sigma}$	= 2.628	2.667	2.689
$3^\circ \boxplus$	"	= 2.623	2.596	2.609

Thus, although the mean value of the precision with which the end-point is obtained is only slightly greater when the field is subdivided (by factors of 1.012, 1.178, and 1.202 as t_L is made 0.10, 0.50, and 0.90 respectively), the scatter of σ_1/I_m is decidedly lessened, by factors (in the same order) of 1.143, 1.346, 1.754. We do not regard the almost rectilinear increase of these factors with t_L to be necessarily accidental, but rather as pointing the way to a distinctly promising mode of inquiry.

The level of $F_{max.}$ (cf. Fig. 3) changes very little with t_L for the subdivided

³⁰ 1940-41, *J. Gen. Physiol.*, **24**, 635; 1941-42, **25**, 89, 293.

square, and the values of r do not change (0.97, 0.96, 0.98); they seem a little higher than with the simple square of field (i), 0.96, 0.95, 0.96, but we cannot hold that such differences are significant. A wider range of tests is necessary before we can conclude that (in the range of F_{max} , where, as in the experiments with fields (i) and (ii), r is ordinarily at a maximum) an increase of the ("cone") slope constant without much change of F_{max} , can affect the value of r . We can say, however, that for the same $\sigma'_{log I}$ the contours with field (iii), in section V, and field (ii) give a higher value of r for the field (ii) with lower F_{max} .

It is clear that these variational constants provide one means of estimating in a simple way the influence of additional factors affecting the internal coherence of data in an otherwise homogeneous set. For example, if conditions are found which permit the comparison of flicker curves brought to the same F_{max} , by choice of t_L , and to the same slope by choice of image area¹⁵, r can be computed under the influence of lowered O_2 pressure, medication, subdivision of the image, and the like. It should provide an index of the relative coherence of the state of neural integration governing the determination of the response. The properties of r confirm in an independent way the multivariate character of the situation controlling visual end-points, and illustrate once more why it is futile to found interpretive conceptions upon data derived from any single set of "standard" circumstances.

V

Systematic investigation of the origin of effects of the type discussed in section III has been inviting. Certain steps have been taken in this direction.¹³ Thus we have ascertained that the simple subdivision of a *small* field into two parts does not necessarily change the flicker contour if this is done in such a way as to produce only a small increase in the extent of the light/dark margin on the field. This could be pursued further. The promising but complex analytical possibilities presented by the flickering of a field in which parts are illuminated by one intensity while other parts are maintained at a different intensity (or λ) may be mentioned. A firm approach to questions posed by the Gestalt psychology of perception is, of course, thus possible.

We are now concerned, however, with the exploration of the nature of the "pecten effect" in flicker.³ The experiment discussed in section III has shown that mere subdivision of an illuminated field periodically illuminated by light sectoried at a focus can cause the "cone" $F - \log I$ curve to be steeper, change its relation to the light-time fraction t_L in the flash cycle, and alter its dependence of F_{max} on t_L . The simple splitting of the field (section III) does not, however, introduce into the dependence of the $F - \log I$ contour on t_L the other major element of what we have recognized as the "pecten effect," namely the thorough upsetting of the normally simple, rectilinear dependence of τ' on t_L .³ This feature is, however, brought in, in a way which we have found sur-

prising, by a further elaboration of the kind of experiment concerned in section III.

An illuminated field of $10^\circ \times 10^\circ$ boundary at the retina was crossed by vertical (or horizontal) stripes. The field was centered at the fovea. Two such fields, already referred to as (iii) and (iv), are mainly concerned here. On (iii) there were three dark and four light vertical bars, each 1.43° wide. On (iv) there were six dark and seven light vertical bars, each 0.77° wide. The

TABLE III

Data for flicker response contours with different light-time proportions, using a foveally centered square test-field subtending 10° on a side at the retina, but divided by *three* equally spaced vertical opaque bars 1.43° wide, the four light bars thus produced being also 1.43° broad. White light, W. J. C., left eye; $n = 10$ at each point.

F per sec.	$t_L = 0.10$ log I_m log P.E. ₁	0.25 log I_m log P.E. ₁	0.50 log I_m log P.E. ₁	0.75 log I_m log P.E. ₁	$p.90$ log I_m log P.E. ₁
2			$\bar{6}.1900$ $\bar{8}.9123$		
4			$\bar{6}.4344$ $\bar{8}.8700$		
6	$\bar{7}.4176$ $\bar{9}.8307$		$\bar{6}.7322$ $\bar{7}.0812$		$\bar{7}.9388$ $\bar{8}.4068$
8	$\bar{7}.7338$ $\bar{8}.1844$		$\bar{5}.0249$ $\bar{7}.3426$		$\bar{6}.2674$ $\bar{8}.6642$
10	$\bar{6}.0434$ $\bar{8}.4604$	$\bar{6}.9098$ $\bar{7}.3605$	$\bar{5}.3606$ $\bar{7}.7894$	$\bar{6}.4251$ $\bar{8}.8221$	$\bar{6}.6029$ $\bar{7}.0178$
12	$\bar{6}.3406$ $\bar{8}.5274$		$\bar{5}.6693$ $\bar{7}.9193$		$\bar{6}.8882$ $\bar{7}.0638$
15	$\bar{6}.5938$ $\bar{8}.9686$		$\bar{5}.8991$ $\bar{6}.2488$		$\bar{5}.1271$ $\bar{7}.3971$
18	$\bar{6}.9853$ $\bar{7}.0384$		$\bar{4}.3064$ $\bar{6}.6410$		$\bar{5}.5350$ $\bar{6}.0799$
20	$\bar{5}.2572$ $\bar{7}.5541$	$\bar{4}.1467$ $\bar{6}.5627$	$\bar{4}.5186$ $\bar{6}.7621$	$\bar{5}.5690$ $\bar{6}.0889$	$\bar{5}.7756$ $\bar{6}.2280$
			$\bar{4}.6005$ $\bar{6}.8459$		$\bar{5}.7806$ $\bar{6}.1131$
25	$\bar{5}.6281$ $\bar{6}.0544$		$\bar{4}.9440$ $\bar{5}.2466$		$\bar{4}.1526$ $\bar{6}.5783$
30	$\bar{5}.9802$ $\bar{6}.3294$	$\bar{4}.8710$ $\bar{5}.1851$	$\bar{3}.2638$ $\bar{5}.6750$	$\bar{4}.2830$ $\bar{6}.7527$	$\bar{4}.5349$ $\bar{6}.8808$
35	$\bar{4}.3267$ $\bar{6}.7527$		$\bar{3}.6229$ $\bar{4}.0678$		$\bar{4}.8620$ $\bar{5}.3466$
40	$\bar{5}.6985$ $\bar{6}.9240$	$\bar{3}.5544$ $\bar{5}.7266$	$\bar{3}.9609$ $\bar{4}.6070$	$\bar{3}.0338$ $\bar{5}.5013$	$\bar{3}.2014$ $\bar{5}.6725$
45	$\bar{3}.0086$ $\bar{5}.2982$		$\bar{2}.3066$ $\bar{4}.6791$		$\bar{3}.5714$ $\bar{4}.0929$
48	$\bar{3}.2567$ $\bar{5}.7830$		$\bar{2}.5824$ $\bar{4}.9849$		$\bar{3}.8418$ $\bar{4}.1857$
50	$\bar{3}.5993$ $\bar{4}.0757$	$\bar{2}.4870$ $\bar{4}.8879$	$\bar{2}.9034$ $\bar{3}.4385$	$\bar{3}.9527$ $\bar{4}.4924$	$\bar{2}.1861$ $\bar{4}.6021$
52	$\bar{3}.9862$ $\bar{4}.3947$		$\bar{1}.2931$ $\bar{3}.7900$		$\bar{2}.5565$ $\bar{3}.0793$
54	$\bar{1}.3251$ $\bar{3}.8437$		0.6188 $\bar{1}.0810$		$\bar{1}.6068$ $\bar{3}.9226$
55	1.5828 0.0721				3.0951 1.6155

idea was to have, within the same total extent of image field, about the same^e illuminated area but about twice the dark/light perimeter in one field as in the other. It will be understood that in an exact sense these several conditions^s cannot really be satisfied. But it will also be apparent that for the purposes^s of the present account they are satisfied to an approximation which is sufficient.

It appeared in the course of the observations that when t_L was variously adjusted something peculiar was involved. This became the subject of very careful tests as to the possible existence of different kinds of flicker end-points

TABLE IV

Data for flicker response contours with different light-time proportions using a square foveally centered test-field subtending 10° on a side at the retina, but divided by *six* equally spaced vertical opaque bars 0.77° wide, the seven light bars thus produced being also 0.77° broad. White light, W. J. C., left eye; $n = 10$ at each point.

<i>per sec.</i>	$t_L = 0.10$ $\log I_m$ $\log P.E._1$	0.25 $\log I_m$ $\log P.E._1$	0.50 $\log I_m$ $\log P.E._1$	0.75 $\log I_m$ $\log P.E._1$	p.90 $\log I_m$ $\log P.E._1$
2		$\bar{6}.3166$ $\bar{8}.7111$	$\bar{6}.6566$ $\bar{8}.8993$ $\bar{6}.6427$ $\bar{8}.8284$		$\bar{6}.1430$ $\bar{8}.3939$
4		$\bar{6}.5527$ $\bar{8}.8319$	$\bar{6}.9909$ $\bar{7}.2377$	$\bar{6}.0820$ $\bar{8}.4342$	$\bar{6}.3836$ $\bar{8}.7004$
5			$\bar{5}.1473$ $\bar{7}.4032$		
6	$\bar{6}.0265$ $\bar{8}.2884$	$\bar{6}.9117$ $\bar{7}.5147$	$\bar{5}.2891$ $\bar{7}.5574$	$\bar{6}.3901$ $\bar{8}.7887$	$\bar{6}.6766$ $\bar{7}.0489$
8	$\bar{6}.3390$ $\bar{8}.6692$	$\bar{5}.2420$ $\bar{7}.5443$	$\bar{5}.5747$ $\bar{7}.7601$	$\bar{6}.7232$ $\bar{7}.1889$	$\bar{6}.9923$ $\bar{7}.1615$
9			$\bar{5}.7813$ $\bar{6}.0305$		
10	$\bar{6}.6980$ $\bar{7}.0014$	$\bar{5}.6909$ $\bar{6}.1366$	$\bar{4}.0212$ $\bar{6}.2638$ $\bar{4}.0993$ $\bar{6}.4370$ $\bar{4}.0094$ $\bar{6}.2076$	$\bar{5}.0715$ $\bar{7}.4736$	$\bar{5}.3641$ $\bar{7}.6642$
12	$\bar{6}.9699$ $\bar{7}.2736$	$\bar{5}.9602$ $\bar{6}.3093$	$\bar{4}.3688$ $\bar{6}.6823$ $\bar{4}.2591$ $\bar{6}.5297$	$\bar{5}.3243$ $\bar{7}.6220$	$\bar{5}.6325$ $\bar{7}.9719$
15	$\bar{5}.3183$ $\bar{7}.6981$	$\bar{4}.2955$ $\bar{6}.7096$	$\bar{4}.6195$ $\bar{6}.9507$ $\bar{4}.6345$ $\bar{6}.8783$ $\bar{4}.6168$ $\bar{6}.8442$	$\bar{5}.6829$ $\bar{6}.0879$	$\bar{5}.9820$ $\bar{6}.4078$
17			$\bar{4}.8774$ $\bar{5}.2219$		
18	$\bar{5}.7124$ $\bar{6}.0083$	$\bar{4}.7033$ $\bar{5}.2473$	$\bar{3}.0374$ $\bar{5}.3021$ $\bar{3}.0191$ $\bar{5}.2923$	$\bar{4}.0842$ $\bar{6}.5098$	$\bar{4}.3820$ $\bar{6}.6847$
20	$\bar{5}.9585$ $\bar{6}.2042$ $\bar{5}.9699$ $\bar{6}.4996$	$\bar{4}.9405$ $\bar{5}.3635$ $\bar{4}.9138$ $\bar{5}.2884$	$\bar{3}.2925$ $\bar{5}.5387$ $\bar{3}.2595$ $\bar{5}.4550$ $\bar{3}.2579$ $\bar{5}.6329$ $\bar{3}.2993$ $\bar{5}.5498$ $\bar{3}.2472$ $\bar{5}.5122$	$\bar{4}.3632$ $\bar{6}.5387$ $\bar{4}.3841$ $\bar{6}.5584$	$\bar{4}.6221$ $\bar{6}.7950$ $\bar{4}.6294$ $\bar{6}.9695$
25	$\bar{4}.3340$ $\bar{5}.7595$ $\bar{4}.3591$ $\bar{6}.6989$	$\bar{3}.2655$ $\bar{5}.5813$ $\bar{3}.2653$ $\bar{5}.6839$	$\bar{3}.6965$ $\bar{5}.9429$ $\bar{3}.6145$ $\bar{5}.9546$ $\bar{3}.6142$ $\bar{5}.8637$ $\bar{3}.6188$ $\bar{4}.0330$	$\bar{4}.7423$ $\bar{5}.0857$ $\bar{4}.7570$ $\bar{5}.1854$	$\bar{3}.0441$ $\bar{5}.3861$ $\bar{3}.0693$ $\bar{5}.4108$
30	$\bar{4}.7560$ $\bar{5}.1824$	$\bar{3}.6502$ $\bar{5}.9995$	$\bar{2}.0519$ $\bar{4}.2574$ $\bar{2}.0580$ $\bar{4}.1879$ $\bar{2}.0374$ $\bar{4}.3021$ $\bar{2}.0195$ $\bar{4}.1752$	$\bar{3}.1007$ $\bar{5}.5098$	$\bar{3}.3936$ $\bar{5}.7681$
35	$\bar{3}.1222$ $\bar{5}.3001$	$\bar{2}.0077$ $\bar{4}.4159$	$\bar{2}.3802$ $\bar{4}.7288$ $\bar{2}.3829$ $\bar{4}.6311$ $\bar{2}.4591$ $\bar{4}.8096$	$\bar{3}.4673$ $\bar{5}.8637$	$\bar{3}.7553$ $\bar{4}.0038$
40	$\bar{3}.4286$ $\bar{5}.8420$ $\bar{3}.3051$ $\bar{5}.6549$	$\bar{2}.3251$ $\bar{4}.7956$ $\bar{2}.3084$ $\bar{4}.6060$	$\bar{2}.7171$ $\bar{3}.0094$ $\bar{2}.7254$ $\bar{3}.0513$ $\bar{2}.7142$ $\bar{3}.0506$ $\bar{2}.7101$ $\bar{3}.0038$	$\bar{3}.7918$ $\bar{4}.1658$ $\bar{3}.7824$ $\bar{4}.2599$	$\bar{2}.0338$ $\bar{4}.4063$ $\bar{2}.0599$ $\bar{4}.4468$

TABLE IV—Continued

F per sec.	$t_L = 0.10$ $\log I_m \log P.E.1$	0.25 $\log I_m \log P.E.1$	0.50 $\log I_m \log P.E.1$	0.75 $\log I_m \log P.E.1$	0.90 $\log I_m \log P.E.1$
45	3.7416 4.1518	2.6302 4.9781	1.0526 3.3986 1.0500 3.3955 1.0704 3.1647	2.1486 4.6041	2.4055 4.7208
48	3.9517 4.2028	2.7811 3.0468	1.2499 3.5530 1.2646 3.5773	2.3866 4.7601	2.7186 3.0678
50	2.2997 4.7345	1.0484 3.4123	1.5101 3.6839 1.5156 3.8324 1.5294 3.9554	2.6503 4.9202	2.9983 3.3512
51			1.6170 3.9639		
52	2.6157 4.8669	1.4643 3.8078	1.9312 2.3438 1.9024 2.1994	1.0792 3.3301	1.4041 3.7520
53			0.3008 2.5454	1.9054 2.3228	0.7453 1.5432
54	1.0550 3.3581	0.3740 2.6791	0.6678 1.0812	3.2904 1.5874	
55	0.4794 2.9032	1.3128 1.3301	1.2646 1.4550 1.2521 1.4001 0.9734 1.3766		

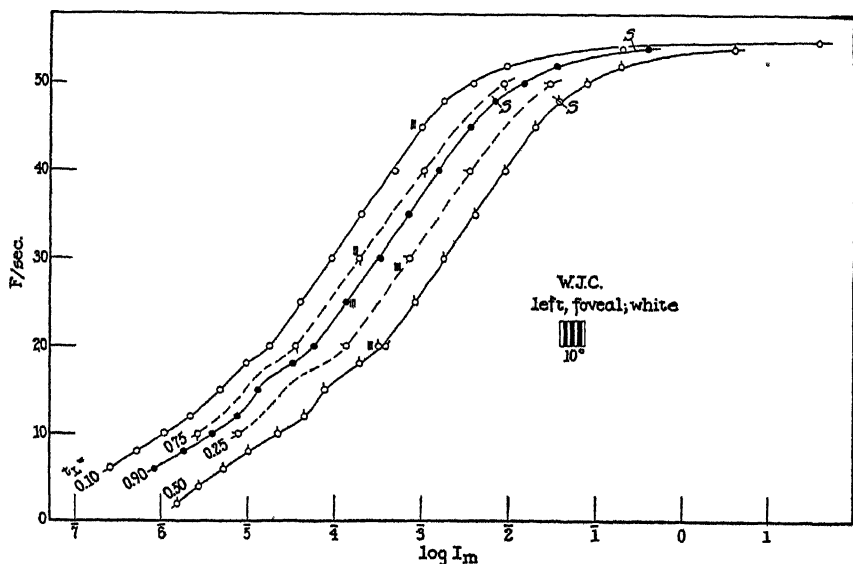


FIG. 7. Flicker contours for a square field 10° on a side over-all, but subdivided into seven vertical bars 1.43° wide alternately illuminated and opaque; centered at the fovea. Note that the curves for $t_L = 0.75$ and 0.90 are at lower intensities than that for $t_L = 0.25$. The contours are quite steep, but clearly of the same general slope. The 3-bar symbol on each curve indicates the level at which the field was recognized as barred. See text. Data in Table III.

which might be confused. We shall return to this presently. But it became clear that this kind of complication did not really enter. The peculiarity had to do predominantly with the location on the $\log I$ axis of the contours at $t_L = 0.75$ and 0.90 . Precisely as in our experiments with moving vertical stripes on a barred field, the contours for $t_L = 0.75$ and 0.90 were shifted bodily to much lower positions on the intensity scale, but in the present case with no change of slope constant and with very slight change of F_{max} . This completes the objective evidence justifying the view that at least three independent parameters are required for the formulation of the (simplex) flicker curve,⁶ since each of our τ' , $\sigma'_{\log I}$, and F_{max} . parameters can thus be experimentally changed in uncorrelated ways. It also appears that three such parameters are sufficient.

The measurements for the contours with fields (iii) and (iv) are collected in Tables III and IV, and are shown in Figs. 7 and 8. The curves for the more finely subdivided field are pitched at a higher intensity level (τ' nearly 1 log unit greater for field (iv)—*cf.* Fig. 10), but show qualitatively the same sort of dependence on t_L . F_{max} . changes more with (iv) as t_L is increased. As shown in Fig. 9 the slope constant $\sigma'_{\log I}$ for (iv) is less than for (ii); *i.e.*, the slope is greater. Here, as in section III, this automatically brings about an increase in the size of the "rod" contribution in (iv), in fact nearly doubles it, although it appears at a higher intensity (*cf.* Figs. 1 and 2). Again we point out that such relationships cannot be rationalized in simple terms of an available population of illuminated retinal receptor units with individually fixed intensity thresholds. In (iv) the illuminated image area (52.9 square degrees) is actually *less* than that (57.2 square degrees) for (iii), although $\sigma'_{\log I}$ is greater, so the "rod" effect cannot be accounted for on this basis.

The lengths of lines of separation between light and dark portions of the barred fields, however, are: (iii) = 91.44° , (iv) = 150.78° , in the ratio 1/1.65. The "cone" slope constants ($1/\sigma'_{\log I}$) are in the ratio 1/1.2. In the experiment of section III, *doubling* the dark/light perimeter increases the slope in the ratio 1/1.43₅.

The very considerable increase in ("cone") slope produced by increasing the subdivision of the field is not confined to white light, but is shown also with separated portions of the spectrum. Data with blue and red lights are given in Fig. 11 (Table V), for $t_L = 0.50$. We have pointed out that with simple fields F_{max} . and τ' occupy an intermediate position for a *white*, as contrasted with the positions (on the same brightness or energy-at-cornea scale for τ') taken by the opposite ends of the spectrum filtered from it.³¹ The properties of $\sigma'_{\log I}$ for different spectral regions are not inconsistent with the view that *white* is not a simple "addition" of primaries, but represents a sort of integrative synthesis.³¹ In general, for simple fields, the $F - \log I$ contour at the same t_L is with *white*

³¹ 1941-42, *J. Gen. Physiol.*, **25**, 89, 293; 1943-44, **27**, 119.

intermediate (τ') between that for *blue* and *red*. The data of Fig. 11 show that by suitable subdivision of the test-field this order can be radically disturbed. On a scale of brilliance intensity the steady light values for the flash intensities adequate to evoke flicker with *blue*, *red*, *white* assume a different order when the field is suitably subdivided. This is also true if the intensities are put on an

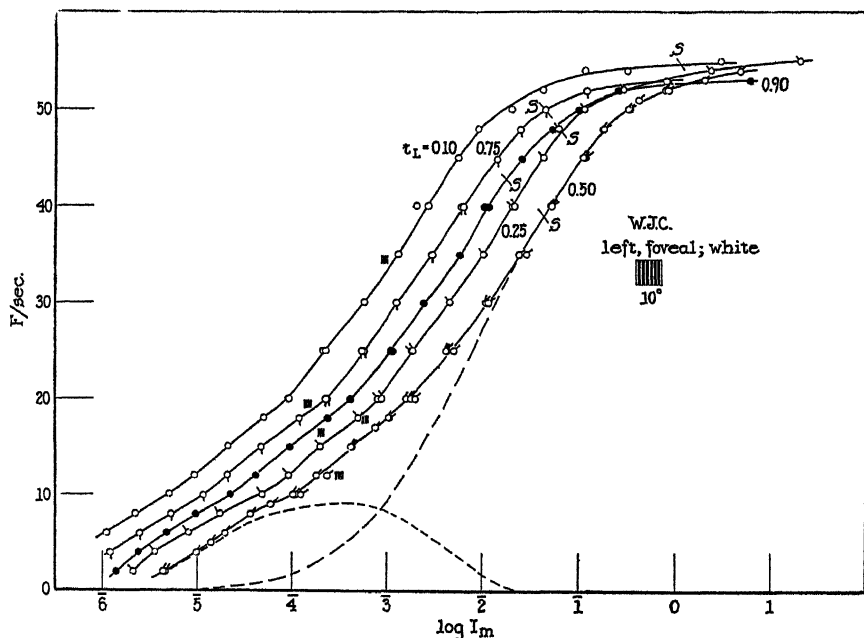


FIG. 8. As in Fig. 7, a 10° square field centered at the fovea, but divided into thirteen alternating light and dark vertical bars 0.77° wide. The curves are steeper than those of Fig. 7 (*cf.* Fig. 9). Data in Table IV. The contours at $t_L = 0.75$ and 0.90 are, as in Fig. 7, so shifted that they fall between those for $t_L = 0.10$ and 0.25 . By comparison with the curves of Fig. 7 those here shown are located at higher intensity levels (*cf.* Fig. 10). Above $\log I = ca. 4.6$, the illuminated fields are speckled. The intensities (barred symbol) at which the barred pattern is resolved, on the contour, are, however, about the same as in Fig. 7. At and above the flash intensities marked *S* the illuminated bars are smooth.

energy basis. At this particular value of $t_L (= 0.50)$, τ for *B*, *W*, *R* is respectively 0.99 , 0.97 , 0.95 ; the differences in σ_s are more impressive: 3.372 , 3.699 , 3.784 . These, like the foregoing, agree with the apparent order of F_{max} , while the differences detectable in $\sigma'_{\log I}$ are insignificant. It is not without value for the conception of the multivariate control of the quantitative properties of the flicker contour that comparatively simple subdivision of the field should alter the order of relative effectiveness of different wave length zones.

According to the view required by the data of the flicker contours we have supposed that (section III) a primary part may be played by the existence of lines of separation between light and dark areas in the flickered field. The striking modifications in the $F - \log I$ curves by simple subdivision of the image area are consistent with this notion. We have been required to assume that the existence of contrast edges, even when not visually resolved, serves to enhance the number of neural units concerned in the discrimination of

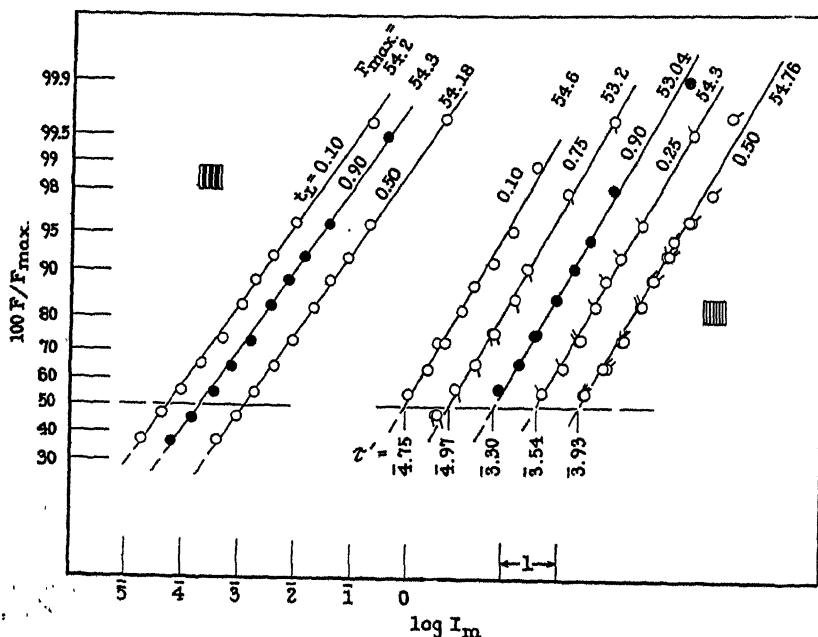


FIG. 9. The higher intensity segments of $F - \log I$ contours in Figs. 7 and 8, shown on a probability grid. Only the upper 50 per cent or so is uncomplicated by the "rod" contribution, but it is significant that for each set the slopes are uniform.

flicker, although total illuminate area is the same, and even though the whole field concerned subjectively flickers uniformly at the critical intensity.

In the data of our fields (iii) and (iv) it has to be emphasized that, as in the case of field (iii) (section III), the effect in question is definitely at work at flash intensities, let alone at brightnesses-at-fusion, well below those adequate for subjective recognition of the fact that the field is actually subdivided. At the moment, there are two chief aspects of interest in this situation. The first has to do with the argument for the essential unity of the organic mechanism determining the form of the flicker contour despite the manifestations of visual duplexity. We have already spoken of the importance of this conception as

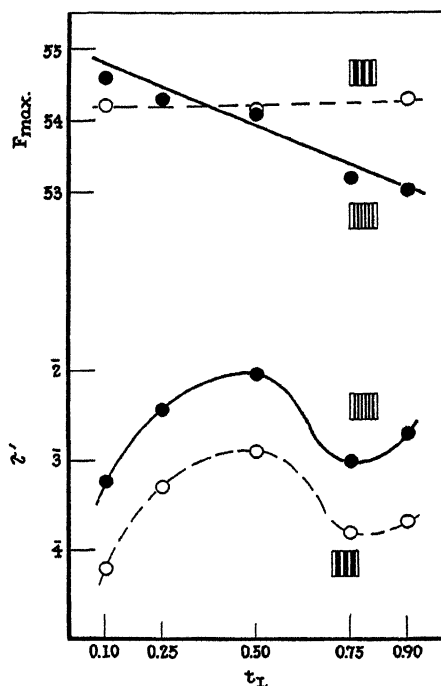


FIG. 10. The values of $F_{max.}$ and of the abscissa of inflection r' obtained from Fig. 9 are shown as functions of t_L , for the curves of Figs. 7 and 8. The structure of the flickered fields is indicated by the barred symbols.

TABLE V

Conditions as in Table IV, but using *red* light (two independent series) and *blue*; $t_L = 0.50$. (Intensities in millilamberts, by matches with white.)

F per sec.	$t_L = 0.50$ Red		$t_L = 0.50$ Blue	
	$\log I_m$	$\log P.E._1$	$\log I_m$	$\log P.E._1$
20	$\bar{4}.6415$	$\bar{5}.1117$	$\bar{5}.1172$	$\bar{7}.4667$
	$\bar{4}.6598$	$\bar{6}.9748$		
25	$\bar{3}.0020$	$\bar{5}.4241$	$\bar{5}.5097$	$\bar{7}.9051$
	$\bar{4}.9983$	$\bar{5}.2916$		
30	$\bar{3}.4240$	$\bar{5}.7272$	$\bar{5}.9501$	$\bar{6}.2187$
	$\bar{3}.4309$	$\bar{5}.8960$		
35	$\bar{3}.8197$	$\bar{4}.1343$	$\bar{4}.2758$	$\bar{6}.5901$
	$\bar{3}.8101$	$\bar{4}.3240$		
40	$\bar{2}.1019$	$\bar{4}.3261$	$\bar{4}.5397$	$\bar{6}.8716$
	$\bar{2}.1366$	$\bar{4}.5074$		
45	$\bar{2}.4295$	$\bar{4}.8989$	$\bar{4}.8576$	$\bar{5}.2005$
	$\bar{2}.4368$	$\bar{4}.9247$		
48	$\bar{2}.6639$	$\bar{3}.0714$	$\bar{3}.0820$	$\bar{5}.4354$
50	$\bar{2}.9555$	$\bar{3}.1484$	$\bar{3}.3669$	$\bar{5}.6639$
52	$\bar{1}.4466$	$\bar{3}.9721$	$\bar{3}.7820$	$\bar{5}.1351$
54	0.2972	$\bar{2}.7095$		
55			$\bar{1}.3418$	$\bar{3}.7976$

rationalizing the quantitative behavior of the scotopic section of the curve under various conditions both mild and drastic. The second aspect of theoretical importance concerns the relations of flash intensity, flash brightness, and especially of fused (Talbot) brightness in flickered light to "visual acuity." This is important in a number of ways which should have been examined a good while ago but seem to have been ignored. We are concerned here to indicate that the data provide a method for the experimental separation of effects due to subjective *brightness* from those due simply to *intensity*. Thus, on the curves of our "pecten" experiment³ the cross-barring of the field was perceptible at the same *flash intensity* down to a light-time fraction of 0.25 (E. W.) or 0.50

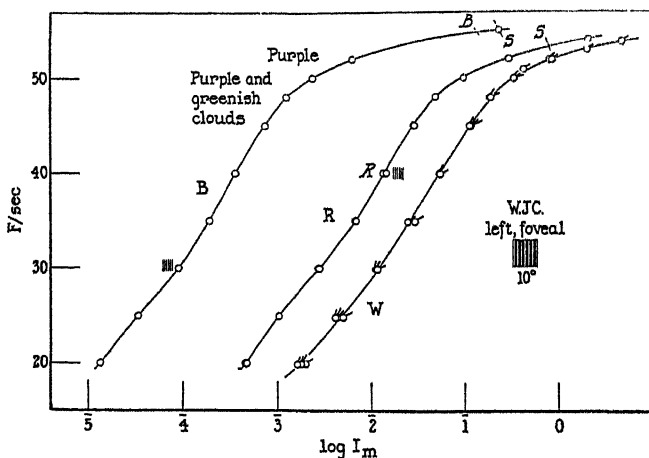


FIG. 11. Flicker contours, $t_L = 0.50$, with the 10° square subdivided into seven illuminated strips, centered at the fovea, for *blue* and *red* (Table V), compared with *white* on the same (steady light) brightness scale. The points at which the illuminated fields become subjectively smooth, exhibit color, and are visually resolved as barred, are indicated.

(W. J. C.). In the experiment of section III the bars subdividing the field were just visible (on the contours) at a flash intensity of 4.80 log units (millilamberts), regardless of the light-time fraction, and thus independent of the fused brightness. (Incidentally, for $t_L > 0.50$, this means that in the tests with the moving stripes³ the field is resolved at the *same* flash intensity regardless of whether there is any sign of involvement of the "cone" curve.) Consequently, we are dealing primarily with effects due to the physical intensity of a flash, rather than to its duration or to the subjective brightness level. For comparatively simple fields, the constancy of the flash intensity for visual resolution in flickered light³² extends to light-time fractions below 0.10, although for more

³² 1943-44, *J. Gen. Physiol.*, **27**, 119.

complex fields this constancy may fail somewhat below $t_L = 0.25$, while even so not obeying the course of the Talbot brightness. A more extensive examination of this matter has been made, and will be described elsewhere. Fields (iii) and (iv), with four and seven illuminated vertical bars respectively, were visually resolved (on the $F - \log I$ contours) at a log flash intensity which declined rectilinearly with increasing t_L (Fig. 12).

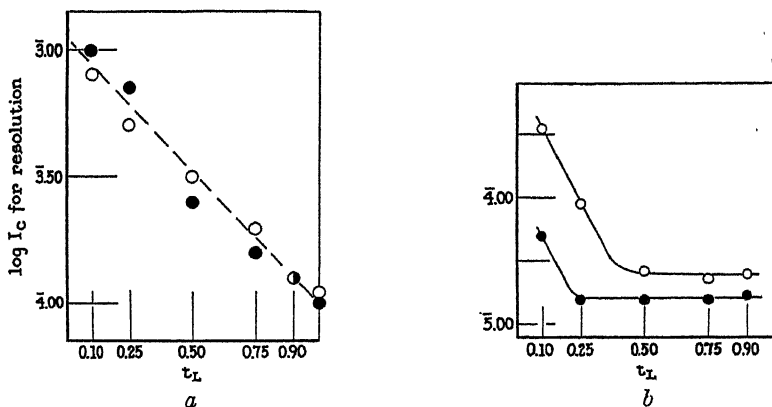


FIG. 12. Illustrating the complex dependence of visual resolution in flickered light upon flash intensity I_c at different light-time fractions, on the flicker contours.

(a) Open circlets, 3-barred pattern (field (iii)); solid circlets, 6-barred pattern (field (iv)). The mean flux, corresponding to the Talbot brightness, is very nearly approximated at the point of resolution; thus reducing t_L to 0.10 requires nearly ten times the flash intensity necessary with steady light.

(b) Flash intensity *vs.* t_L for the resolution of the barred pattern of our "pecten" experiment;^{2,3} open circlets, W. J. C.; solid dots, E. W. Here, over a good range, resolution of the pattern is determined by flash intensity alone, independent of t_L .

Thus there are conditions under which resolution of a more finely subdivided field is possible with a lower flash intensity, even when the length of the subdivisions is the same. The rôle of the latter factor is easily demonstrated.³³ It has been known³⁴ that the presence of a nearby contrast border can affect the visual resolution of a given such border. The flicker data prove that phenomena of this kind, involving pronounced integration of visual functioning, can operate even when contrast as such is not perceived (*i.e.*, below the flash intensity required for resolution of the barred pattern).

One aspect of the integrative action of an increased number of effective neural units is reflected in the associated variational indices, as we have already sug-

³³ A following paper deals with certain of these questions.

³⁴ Cf. Bartley, S. H., 1941, *Vision: a study of its basis*, New York, D. Van Nostrand Co., Inc., Chapter X.

gested. In the data of Tables III and IV, σ_1/I_m is statistically constant. The values for $\bar{\sigma}$ and for σ_s here differ relatively little, although those for the 4-bar field (iii) may be slightly higher. They are smaller, however, than one would be led to expect for a simple field of the same illuminated area. For a square 6° field at the fovea, the values of $\bar{\sigma}$ are not different significantly, but those for σ_s are larger with the 10° field by the average factor of 1.74, although the fields (iii) and (iv) are half again as large, and F_{max} is decidedly higher, and we find that σ_s increases when the image area of a simple square is enlarged in this way. The constant r is consistently larger by a little for the 7-bar field (iv) than for the 4-bar field (iii), the latter having less than two-thirds of the light-dark perimeter in the former. (Fig. 13.) This is quite striking because of the general relation previously described for simple fields between F_{max} and r .

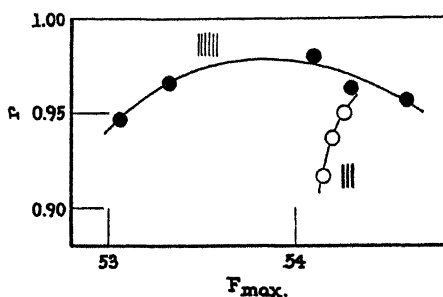


FIG. 13. The values of r in the expression $\sigma_s/\bar{\sigma}\sqrt{2} = \sqrt{1-r}$ (see text), as a function of F_{max} , for the series of flicker contours with fields (iii) and (iv).

We conclude, therefore, that the degree of integration involved in the recognition of visual flicker, as a function of flash frequency and intensity, is decidedly enhanced by the imposition of image forms (subdivision into stripes) such that more visual units are involved together with a reduction in the effective contribution of elements of neural action from each unit.

VI

The peculiar transposition of the t_L 0.75 and 0.90 contours in the data of section V led us to inquire if we were being in some fashion misled by the occurrence of more than one intensity critical for subjective flicker, at a given F . It is true that under certain conditions $t_L = 0.50$ is a critical light-time fraction, as Porter³⁵ found for fixed illumination reflected from a spun sector disk; this result, for which an explanation has been offered,³⁶ does not enter here. Be-

³⁵ Porter, T. C., 1898, *Proc. Roy. Soc. London*, **63**, 347.

³⁶ 1937-38, *J. Gen. Physiol.*, **21**, 313, 463; Pieron, H., 1935, *Ann. Psychol.*, **35**, 1.

sides, it is desirable to be able to account for the fact that the curve of τ' as a function of t_L exhibits a minimum at $t_L = 0.75$ (Fig. 10).

In tests of visual acuity with grating patterns it has been found that a difference in resolvability appears when the grating stripes are at different angles to the vertical.³⁷ For our field (iv) we find that this difference likewise appears under flickered light (Table V, Fig. 14), and also that the whole flicker contour is displaced toward slightly higher intensities when the barred field is turned

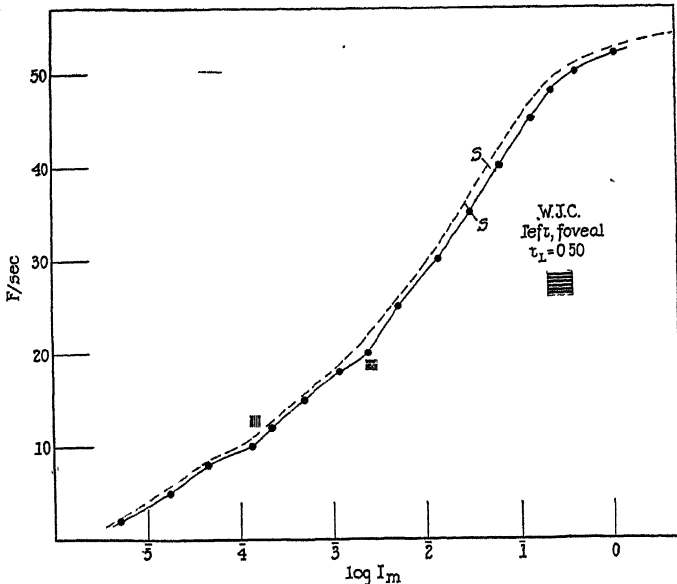


FIG. 14. Flicker contour for 6-barred field (iv) with stripes vertical (dashed line, from Fig. 8, $t_L = 0.50$) compared with that for the same field with the stripes rotated 90° , horizontal.

The point above which the horizontally barred pattern was resolved is indicated by the symbol, well above the level for the vertical bars. At S the illuminated areas became smooth. See text.

with the stripes horizontal (field (v)). We doubt that gross ocular astigmatism can account for this difference, since it persists when the very slight astigmatism is suitably corrected. A more natural explanation may be found in the neural structure of the foveal region ("retinal astigmatism"). This is described as receiving rather distinct upper and lower groups of nerve fibers.³⁸

³⁷ Shlaer, S., *J. Gen. Physiol.*, 1937-38, **21**, 165.

³⁸ Duke-Elder, W. S., *Textbook of ophthalmology*, St. Louis, C. V. Mosby Co., 1938, **1**, 262; Polyak, S. L., *The retina*, Chicago, University of Chicago Press, 1941, Fig. 43.

The suggestion is that flicker may be a little less easily perceived when groups of neighboring receptor units are simultaneously illuminated in the same manner, if their fiber connections remain together. Calculation of the r constant

TABLE VI

Illustrating the relation between flash frequency and flash intensity critical for the appearance of the " γ movement effect;" conditions as in Table V, $t_L = 0.75$. The γ movement thresholds are of course obtained only above flash intensities at which the illuminated bars are separately visible; see text.

F per sec.	$t_L = 0.75$ $\log I_m$ $\log P.E._1$	
20	5.7859	7.9160
25	4.1309	6.4788
30	4.5298	6.9326
35	4.8813	5.1306
40	3.2127	5.6238
45	3.5371	5.8284
48	3.7367	4.0200
50	2.0269	4.3191
52	2.4200	4.5834
54	1.2709	3.5251

TABLE VII

An exceptional series of observations under the conditions specified in Table V for $t_L = 0.50$, involving the " ϕ effect;" see text.

F per sec.	$t_L = 0.50$ $\log I_m$ $\log P.E._1$	
20	3.4763	5.7281
25	3.8308	4.2444
30	2.1959	4.4441
35	2.5572	4.9065
	2.5526	4.7402
40	2.8596	3.1561
45	1.2156	3.5297
	1.2388	3.4890
48	1.5173	3.7681
	1.4965	3.7193
50	1.8799	2.0079
	1.8511	2.1172
52	0.2804	2.5742

from the flicker data shows that it is little, but significantly, lower (0.966 *vs.* 0.980) for the horizontal stripes. The estimated F_{max} is also a little lower. This would indicate an organizational factor, which might of itself be a function of the light-time fraction.

We have sought diligently to see if more than one flicker end-point could be found on the intensity scale, with F and t_L fixed, but we have not found any. It is true that with the barred pattern (iv) visual disturbances of certain interesting kinds are easily recognizable at other critical intensities when the bars are almost or quite resolved, but there is no difficulty in distinguishing them from the occurrence of *flicker*. The most interesting of these we have regarded as an example of " γ " apparent movement. This, most obvious at $t_L = 0.75$, consists in a pulsatile widening and contracting of the illuminated stripes,³⁹ at a rate lower than that of the flashes. The end-point is easily

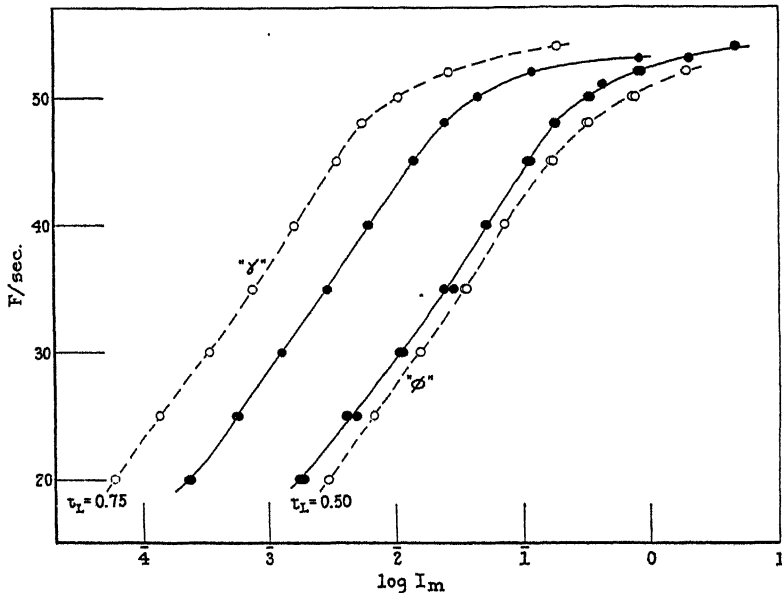


FIG. 15. Upper portions of *flicker* contours for $t_L = 0.50$ and 0.75 , solid circlets, from Fig. 8, with corresponding curves for " γ " movement and " ϕ " movement. See text.

recognized. As shown in Fig. 15, the " γ " effect provides a contour similar to that given by the flicker end-point, although at lower intensities and with a higher F_{max} . (54.5 vs. 53.2 for flicker). Analysis of the curves in Fig. 15 shows that $\sigma'_{\log I}$ is not different for these " γ " data. It is of some interest that for the " γ " set the relative variation (σ_1/I_m) is lower than for flicker at $t_L = 0.75$, by a factor of 0.835; and the scatter of this ratio (σ_σ) is also lower, by the factor 0.867, so that r is not essentially different, being 0.964 vs. 0.966.

The conclusion that the same neural units, to the same number, are con-

³⁹ Cf. Bartley, S. H., 1941, *Vision: a study of its basis*, New York, D. van Nostrand Co., Inc., Chapter VII.

cerned as in the recognition of flicker, although the subjective effect used is not the same, is supported by other series of measurements based upon what we regard as another kind of apparent movement which we may for convenience but without prejudice label the " ϕ " type.³⁹ This sort of "movement" in the subdivided visual field may be described as a kind of pulsatile pseudomovement, running from side to side of the field. We cannot be entirely certain that it is independent of eye movements. It appears at a flash intensity slightly but systematically above the unmistakable flicker end-point. In our experience with field (iv) it is best developed at $t_L = 0.50$, and its use as an end-point gives a contour at slightly higher intensities than for flicker (Fig. 15), although again $\sigma'_{\log I}$ is the same; $F_{max.}$ is a little lower than for flicker at the same t_L (53.05 vs. 54.16); σ/I_m is a little lower, σ_σ still lower, r is the same (0.977 v. 0.980). It may be remarked that, just as for flicker when t_L is varied, τ' and $F_{max.}$ tend to change reciprocally.

In connection with the relation of (critical) flicker to other subjective phenomena we should note that a certain independence of the properties of the contours can be demonstrated as concerns brightness,²⁵ visual resolution of the field structure,³² color,⁴⁰ and other phenomena. Thus, in Figs. 2, 7, 8, and 14 points along the contours are indicated at which the respective fields are no longer "speckled" or "frosted" but become "smooth" and evenly illuminated; so also in Fig. 11 these points are shown, and the color thresholds. For the " γ " curve of Fig. 15 the light bars appear smooth only above $\log I_m = ca. 1$, and the bars are visually resolved at about $\log I_m = 4.0$, just as for the normal flicker end-point at $t_L = 0.75$. The levels of occurrence of these effects, while related in interesting ways to t_L , are not associated in any manner with the presence of singularities on the $F - \log I$ curves.

To account for the relative positions of the t_L contours with the barred fields we must turn to properties of the "edge effect." The reality of this effect is attested (1) by the systematic changes in the shapes of the curves which cannot be accounted for by changes in image area (sections III and V), and (2) by properties of the variational indices. It is also consistent with the fact that, subjectively, arrival at the critical intensity produces a more pronounced flicker along the image edges (although the whole illuminated area flickers simultaneously). To account for the non-specific nature of the shifts of $F - \log I$ curves with change of temperature and of t_L it has been proposed⁴¹ that the flash intensity critical for flicker results from the appropriate relation between the effects of flashes and of the decay of their after-effects. With the barred fields we have indication that the number of neural units concerned is large ($\sigma'_{\log I}$ is small). The fact that $F_{max.}$, although not very high, changes so very little with t_L (as with field (ii) also) must be taken to mean that, although the

⁴⁰ 1941-42, *J. Gen. Physiol.*, **25**, 89, 293, 369; 1943-44, **27**, 119.

⁴¹ 1936-37, *J. Gen. Physiol.*, **20**, 393; 1937-38, **21**, 313.

mean contribution of elements of effect per neural unit is small it tends to decrease with t_L less than might be expected. It is known that the relative expansion and contraction of an illuminated bar in γ apparent movement is a function of t_L , and we have already pointed out that with our fields (iii), and particularly (iv), it is a maximum (in our series) at $t_L = 0.75$. The conception then might be that the "edge" or bar effect with which γ apparent movement is associated, and of which the latter is one expression, operates in such a way as to contribute elements of effect, from the same units, in addition to those ordinarily concerned in the recognition of flicker, and that this contribution is more marked above $t_L = 0.50$. To the extent that, let us say, a symmetrical balance of the expanding and decaying phases of the γ apparent movement must be achieved for effective reinforcement of the ordinary flicker effect, this could well result in a lower critical intensity for the net result with a given F at the level of t_L most efficient for the " γ " effect. Ordinarily, with simple (rectangular) fields, above an image area of *ca.* 0.73 square degrees, the change of τ' per unit change of t_L ($= \Delta \tau' / \Delta t_L$) is found to decrease steadily as the image area is increased.¹³ This is correlated with changes in σ' and in F_{max} , signifying an increase in number of units concerned and a relative diminution in the number of elements per unit required to evoke flicker. In our field (ii) $\Delta \tau'$ is considerably reduced by comparison with (i), which is consistent with the foregoing. For the striped fields (iii) and (iv) the behavior of τ' seems to show that the number of elements of effect per neural unit required to be involved at the end-point is reduced by the "edge effect," especially at lower values of t_L . The " γ " phenomenon appears to reverse the change of τ' with increase of t_L when it becomes a sufficient factor just beyond $t_L = 0.50$. At $t_L = 0.90$ the " γ " effect is subjectively not so pronounced, under our conditions; a lesser contribution from it is thus to be expected, and therefore τ' must increase in the usual way, relative to the 0.75 contour.

This interpretation is of course tentative, but some interesting tests can be based upon it.

VII

SUMMARY

Flicker contours for a square image of 3° visual angle, centered 6° on the temporal side of the fovea, the light sectorized at a focus, are strikingly modified if the same illuminated area is arranged in four squares separated by a narrow opaque cross. The "cone" curves are made much steeper, and their abscissae of inflection (τ' are at higher intensities; F_{max} is not greatly changed, but alters less with change of light-time fraction in the flash cycle (t_L). This modification is accompanied by a great enlargement of the scotopic segment of the duplex curves, consistent with the theory of the integrative relations of neural effects in the two groups of units involved. The changes are not consistent with the

view that flicker end-points are determined by the activation of retinal cells with a fixed spatial distribution of invariable thresholds. At $t_L = 0.50$ the 3° subdivided area gives very nearly the same contour as does a square $6^\circ \times 6^\circ$, with the same total perimeter of light-dark separation; the "edge effect" thus suggested is complicated by differences in the dependence of F_{max} and τ' upon t_L .

When an image pattern is produced by a grid of light bars separated by equally broad opaque spaces ($10^\circ \times 10^\circ$ over-all, centered at the fovea), the photopic flicker contours are made very steep and their midpoints are situated at quite low intensities, while the "rod" contribution tends to be more completely fused with the "cone" than is found for fields not subdivided. However, instead of a progressive increase of τ' with t_L the curves for $t_L = 0.75$ and 0.90 lie respectively *below* that for $t_L = 0.25$ and 0.50 for a field of four broader stripes (1.43°) and both are below $t_L = 0.25$ for a field of seven narrower stripes (0.77°). These latter changes are discussed in terms of the participation of subsidiary phenomena involving so called " γ movement."

It is pointed out that since in these data σ_1/I_m is for each set of conditions a statistically constant quantity with a characteristic breadth of scatter σ_σ , it is possible to calculate a "coefficient of internal correlation" r which is a function of the conditions (as: image area, location, wave length of light, structure of image, light-time fraction) and which describes a property of any entire contour. The changes in r , as a function of the conditions of flicker excitation, reflect changes in the neural organization responsible for the liminal discrimination of flicker.

It is shown that as consequence of simple changes in the image field, three parameters, as of the probability summation, are required for the description of a simplex flicker contour—since each of these is independently modifiable as to its magnitude and in its dependence on the light-time fraction.

Subdivision of the image, with light sectoried at a focus, produces in part only the changes in the flicker contour which we have earlier labelled the "pecten effect." In the latter, with light not sectoried at a focus but with bar images moving across a field with inclined fixed opaque bars, the "cone" slope ($dF/d \log I$) is sharply increased for $t_L > 0.50$, but not below $t_L = 0.50$, and the value of τ' is much less than it "should be." Consequently, the change in contrast brought about by the moving contact of light/dark borders is the significant factor in the "pecten effect," not simply pulsatile interruption of the light.

THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

VIII. QUANTITATIVE STUDIES CONCERNING THE ACIDIC PROPERTIES OF COLLODION AND THEIR CORRELATION WITH MEMBRANE STRUCTURE AND ACTIVITY

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I

In preceding papers¹⁻³ it was shown that the electrochemical behavior of collodion membranes in solutions of strong, weakly adsorbable electrolytes is due to the presence of acidic impurities in the collodion. Pertinent quantitative data were obtained from base exchange studies.⁴ No regular correlation was found between "electrochemical activity"¹⁻⁴ and base exchange capacity. A measurable base exchange capacity seems to be associated always with good or high electrochemical activity; but base exchange capacities too low to be definitely measurable with the methods used were found with collodion preparations of high as well as with low electrochemical activity.

The very low base exchange capacities even of very active preparations could be due to a very low content of acidic groups or to a lack of availability for base exchange of the majority of them. Thus, it became of interest to obtain quantitative information on the equivalent weight of collodion preparations of different electrochemical activity and to compare it with their base exchange capacity. In this way one could hope to obtain valuable information on the relative availability of the acid groups and on the submicroscopic, micellar structure of collodion fibers or collodion membranes from different preparations.

On certain aspects of this problem we already have some information. Work from this laboratory indicates that the base exchange capacity corresponds to but a fraction of all the acid groups present in collodion. Furthermore we already know what the rate at which base exchange occurs varies with different collodion preparations, faster base exchange being indicative of relatively open structure.⁴

¹ Sollner, K., and Abrams, I., *J. Gen. Physiol.*, 1940, **24**, 1.

² Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467.

³ Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **25**, 7.

⁴ Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, **25**, 411.

II

The experimental approach to our problem is quite obvious; a number of collodion preparations of different past history and varying electrochemical activity are brought into the state of free acids by the exchange of all other cations for hydrogen ions, and their acidity—their acid number—in the dissolved state as well as their base exchange capacity in the fibrous state are determined.

The preparations used in the present study were prepared in the same manner as described previously.^{4,5} Electrodialysis for 72 hours did not change the free acidity of these preparations.

For the *base exchange* experiments, as in the prior work, fibrous collodion was employed. The reasons for the use of fibrous collodion were (1) that different preparations can readily be brought into approximately the same physical state, a condition which is necessary for any comparative investigation, (2) that the precipitated collodion fibers exhibit a maximum surface thus yielding the highest obtainable (and thus most easily determined) base exchange for each preparation, (3) that the collodion in this state is brought in a fairly reproducible manner into a condition similar to the state it assumes in highly porous membranes.

The base exchange experiments were carried out essentially as described previously;⁴ in all cases the water blank was negligible.⁴ The double distilled water used for all experiments had a pH of 6.5 to 7.2.

To measure the base exchange 50 ml. of water or 0.5 N potassium chloride solution was added to 1.5 gm. of dry fibrous collodion in 125 ml. Pyrex Erlenmeyer flasks. The samples were shaken for several minutes and allowed to react—unless otherwise stated—for 48 hours with occasional shaking. 10 ml. samples of the supernatant fiber-free liquid were used for the determination of the pH (with a glass electrode) and the same samples were also used for titration.

For the present work we used a somewhat more accurate titration procedure than previously. We made use of a micrometer microburette which allows readings to be made with an accuracy of about 0.001 ml.⁶ Another change was that a glass electrode was used to determine the neutral point instead of phenol red, which was used in previous work. The reagent used for titration was 0.015 N sodium hydroxide. The accuracy of the experimental figures as given below in column 7 of Table I is about ± 0.004 ml. of 0.01 N solution. As previously the results given below in the tables are expressed in milliliters of 0.01 normal solution per gram of dry collodion.

The *acid number* was determined by potentiometric titration of the collodion which was dissolved in a suitable organic solvent as described by Clarke, Wooten, and Comp-

⁵ In the present work the commercial preparations were treated more carefully than previously (Sollner, K., Carr, C. W., and Abrams, I., *J. Gen. Physiol.*, 1942, **25**, 411), for we had found that these preparations which were stored in the laboratory for some time gave off some acidic impurities arising from spontaneous decomposition. Repeated short boiling in the fibrous state with double distilled water satisfactorily removed these impurities.

⁶ Dean, R. B., and Fetcher, E. S., Jr., *Science*, 1942, **96**, 237.

ton;⁷ Wooten and Ruehle;⁸ and Ruehle.⁹ (See also below.¹⁰) The titration is performed with a solution of alkali hydroxide in an organic solvent. The electrode which responds to the change in acidity is a quinhydrone electrode; some quinhydrone is added to the collodion solution, with a bright platinum wire dipping into it. The reference electrode is a calomel half cell which is connected in the usual manner by means of a potassium chloride-agar bridge to the solution. To increase the conductance of the titrated solution some saturated solution of lithium chloride in alcohol is added. Nevertheless, the ohmic resistance of the system is rather high and an electron tube voltmeter must be used. For this purpose we have used the volt scale of a commercial (Leeds and Northrup) glass electrode.

We have tested a number of different solvents and solvent mixtures for their suitability for our special problem. Some of the solvents recommended in the literature are not obtainable in a sufficiently pure state and can be purified only with considerable difficulty. On account of their ready availability in great purity, we finally worked with acetone and absolute alcohol, mixtures of which show excellent solvent properties for collodion.

To obtain satisfactory results, it is necessary to take certain precautions. The quinhydrone should be carefully recrystallized and stored in a dark bottle. The platinum electrode must be heated in a flame prior to each experiment. The lithium chloride solution is prepared by refluxing 200 gm. of the best obtainable grade of the salt with 1 liter of absolute ethyl alcohol; it is stored in a dark bottle in the dark. The reagent used was 0.015 N potassium hydroxide dissolved in absolute ethyl alcohol. It is kept in a blackened Pyrex glass bottle in the dark. Advantageously, the bottle is kept rather full to minimize the influence of the oxygen of the air. Daily supplies are withdrawn from this bottle and kept ready for use in a small bottle. The titer of the potassium hydroxide solution was determined by titration of a known aqueous hydrochloric acid solution. The titer should be checked at frequent intervals, as any change in titer indicates a very disturbing change in the solution, probably due to spontaneous oxidation. The titrations were carried out with a micrometer microburette of the above mentioned type;⁶ a stream of nitrogen bubbles was passed through the solution during the titration to stir the solution and to prevent the interference of carbon dioxide.

The experiments were carried out in 10 ml. of 2.5 per cent collodion solution in a mixture of 50 per cent acetone and 50 per cent absolute ethyl alcohol to which 0.5 ml. of the saturated solution of lithium chloride in alcohol was added. About 20 mg. of quinhydrone were added and nitrogen is bubbled through the solution. The electrodes are inserted into the solution and the potential difference is read on the millivolt scale of the electron tube voltmeter. The reagent is added step-wise and the

⁷ Clarke, B. L., Wooten, L. A., and Compton, K. G., *Ind. and Eng. Chem., Analytical Edition*, 1931, **3**, 321.

⁸ Wooten, L. A., and Ruehle, A. E., *Ind. and Eng. Chem., Analytical Edition*, 1934, **6**, 449.

⁹ Ruehle, A. E., *Ind. and Eng. Chem., Analytical Edition*, 1938, **10**, 130.

¹⁰ For valuable private information we are indebted to Dr. H. M. Spurlin, Hercules Experiment Station, Wilmington, Delaware.

corresponding potential values are determined. The true neutralization point is determined in the conventional graphic manner.

To test the titration method we have carried out some determinations of known concentrations of benzoic and salicylic acid; benzoic acid, undoubtedly, is a weaker acid than the "nitrocellulosic acid" in which we are interested.¹¹

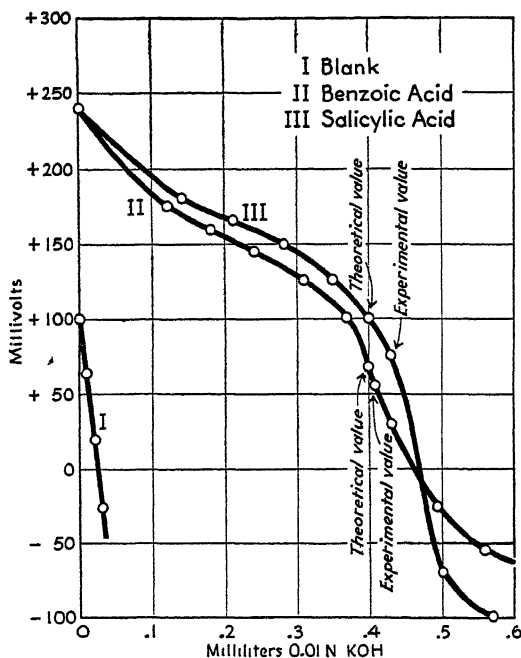


FIG. 1

Sample curves for benzoic and salicylic acid are given in Fig. 1, together with a blank. The latter, as can be seen easily, is negligible.

Sample curves for the titration of various collodion preparations are given in Fig. 2; the ordinate indicates millivolts, the abscissa, milliliters of 0.01 normal solution for 0.25 gm. collodion. The shape of the titration curves for collodion does not allow too accurate an evaluation. An error of ± 5 per cent may occur in many instances. This, however, is of no significance for our particular problem.

Any chemical reaction with the collodion aside from the straightforward

¹¹ The strength of "cellulosic acid," (oxycellulose) is of the same order of magnitude as salicylic acid. (See, *e.g.*, Neale, S. M., and Stringfellow, W. A., *Tr. Faraday Soc.*, 1937, **33**, 881.) One would expect that "nitrocellulosic acid," oxidized nitrocellulose, would be a stronger acid than non-nitrated oxycellulose.

neutralization of its free acid groups is bound to falsify the results. With a material so liable to be decomposed by alkali solutions as collodion it was therefore necessary to test whether or not the reagent added in the process of titration may not act upon the nitrocellulose. This was the more advisable as conceivably the quinhydrone too could be a disturbing factor.

To decide this question, a large sample of a relatively inactive collodion was dissolved in a mixture of 50 per cent acetone and 50 per cent absolute alcohol and titrated with 0.02 N alcoholic potassium hydroxide solution in the presence of the usual con-

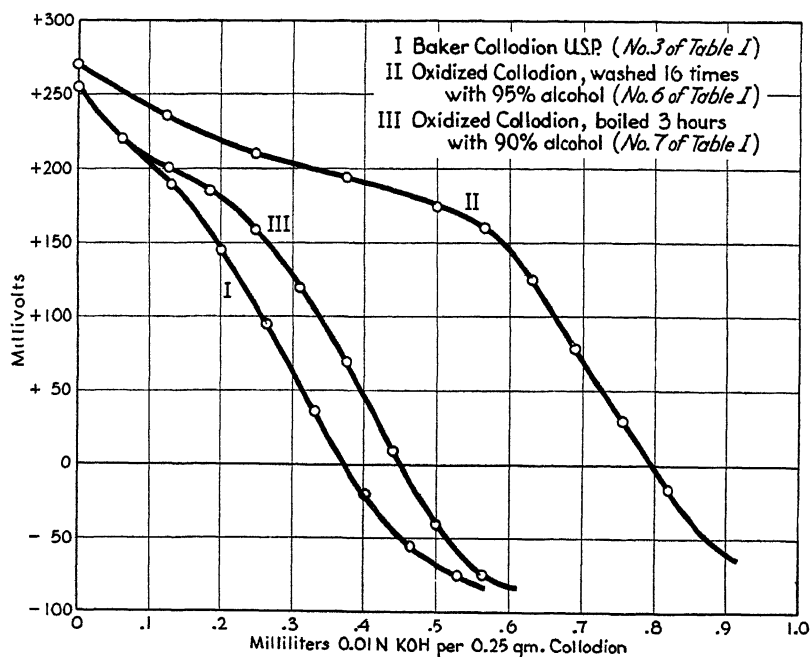


FIG. 2

centrations of lithium chloride and quinhydrone. Next the resulting solution was allowed to drip into a great excess of vigorously stirred water, our standard method of preparing fibrous collodion. The fibrous preparation thus obtained was washed and electro-dialyzed for 24 hours to return it to the state of free acidity. After this the material was dried.

A part of the dried product was again dissolved in the standard mixture of alcohol and acetone and titrated as before. The results of the first and this second titration agreed with each other well within limits of experimental error.

The other part of the collodion which had undergone titration, reprecipitation, electro-dialysis, and drying was dissolved in ether-alcohol. Membranes were prepared from this solution and their activity was compared by our usual anomalous osmosis

method¹⁻⁴ with the activity of membranes prepared from the original material. No difference between the two preparations could be detected.

From the two foregoing experiments it can be concluded that the collodion in the process of titration does not undergo any chemical changes which influence its acid number. Thus we are sure that the acid number as found by titration of collodion in the dissolved state can be considered to be the correct measure of the free acidity of the collodion.¹²

III

In Table I are listed the acid values and base exchange data obtained with some representative preparations of different activity and varying acidity.

Column 2 of Table I gives a brief description of the nature of each preparation.

In columns 3 and 4 are listed the ash and SO_4 contents determined by previously described methods.²

Column 5 gives the acid values expressed in milliliters of 0.01 N hydroxide solution per gram of dry collodion, column 6 the acid values corrected for the base content of the ash.¹³ Below we will see that for our purpose it is actually

¹² We have also tried the following method to determine the acid number of collodion; it was devised originally by Wilbrandt (Wilbrandt, W., *J. Gen. Physiol.*, 1935, **18**, 933) for a similar purpose, though on the basis of entirely different theoretical assumptions, which we have discussed and criticized in an earlier paper (Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467). The method consists of determining the limiting number of equivalents per gram of collodion of basic material which renders the collodion electroneutral. Dried collodion membranes are prepared from a series of collodion solutions containing increasing concentrations of such basic substances as methylene blue or quinine. The concentration of basic materials is determined at which the concentration potential 0.1 M KCl/0.01 M KCl changes its sign. At this point the negative charges of the membrane are neutralized electrically by what is supposed to be an equivalent quantity of base. For a number of reasons such experiments give results which, though in rough agreement with the results of the titration method, are not better than semiquantitative in character. We therefore only mention this method without going into further details.

¹³ The ash content is by no means all basic material; in most cases more than half of the weight of the ash is SO_4 . In one sample we found 26 per cent $\text{Al}_2\text{O}_3 + \text{Fe}_2\text{O}_3$, 7 per cent calcium oxide, and 11 per cent sodium oxide. The aluminum and iron oxides in all probability form but slightly dissociated or non-dissociable compounds. Thus, if we assume that 25 per cent of the weight of the ash behaves like sodium, we certainly have not underestimated the degree to which potentially active acid groups in the collodion are neutralized from the beginning. With an ash content of 0.1 mg. per gm. collodion this would correspond to 0.025 mg. sodium per gm. or about 1 milliequivalent of sodium per kilogram dry collodion. Expressed as are the acid num-

immaterial whether we base our considerations on the uncorrected or the corrected acid number values.

Column 7 indicates the milliliters of 0.01 N hydroxide solution per gram of dry collodion which were used to neutralize 0.5 N potassium chloride solution after 48 hours contact with the collodion.

Column 8 shows the pH values (as determined with a glass electrode) of the potassium chloride solution after 48 hours contact with collodion.

Column 9 gives the base exchange values calculated from the pH values of column 8 under the assumption that the acidity which is found experimentally is caused by hydrochloric acid in an unbuffered system. The difference between the base exchange values as obtained by direct titration (column 7) and those calculated from the pH values (column 9) is a measure of the dissolution of some material from the collodion which reacts with NaOH. The values of column 9 are therefore a more correct expression of the true base exchange than those of column 7. For a discussion of this point we refer to a prior paper.⁴

Columns 10 and 11 indicate the electrochemical activity of the different collodion preparations. The same method of characterization was used as in preceding papers.¹⁻⁴ The membranes tested were approximately of the same porosity, as indicated by their behavior when tested with sucrose solution, the figures in column 10 indicating the millimeter pressure rise observed 20 minutes after the membranes filled with 0.25 M sucrose solution were placed in distilled water. Column 11 gives the anomalous osmotic rise in millimeters of water obtained after 20 minutes with M/512 potassium sulfate solution. As shown previously,^{2,3} the rate of this rise is a rather sensitive indicator of the electrochemical activity of collodion.

Table II gives some representative data, demonstrating the influence of time upon the base exchange. The preparations 3a, 4a, and 6a used for these experiments were not the same but parallel samples of the corresponding preparations Nos. 3, 4, and 6 in Table I.

IV

We turn now to the discussion of the main problems of this paper: (a) the comparison of the acid number of preparations of different electrochemical activity; (b) the comparison of the base exchange capacity of the different preparations in the fibrous state; (c) the comparison between the base exchange capacity and the acid number of the individual collodion preparations; and (d)

bers in Table I, this is 0.1 ml. of 0.01 N sodium hydroxide solution per gram dry collodion.

In calculating the values of column 6 the appropriate additions have been made to the values in column 5. Since the base content of the ash is probably lower rather than higher than the assumed 25 per cent sodium, some of the corrected values of column 6 are probably slightly too high.

TABLE I

Acid Number, Base Exchange, and Activity of Various Collodion Preparations

1	2	3	4	5	6	7	8	9	10	11
	Brand of collodion and pretreatment (All preparations were precipitated from ether-alcohol solutions and dried)	Ash and SO ₄ content		Acid No.		Base exchange data (48 hrs.)			Electrochemical activity	
		Ash per gm. dry collodion	SO ₄ per gm. dry collodion	0.01 N KOH per gm. dry collodion		0.01 N NaOH per gm. dry collodion found experimentally on treatment with 0.5 M KCl	pH values found experimentally on treatment with 0.5 M KCl	0.01 N NaOH per gm. dry collodion, calculated from pH values of column 8	Osmotic rise with 0.25 M sucrose	Anomalous osmotic rise with $\frac{M}{512}$ K ₂ SO ₄
				Experimental	Corrected for ash					
		mg.	mg.	ml.	ml.	ml.		ml.	mm.	mm.
1	Mallinckrodt "Parlodion," commercial preparation	<0.2	<0.1	1.0	1.2	0.011	6.3	0.0016	110 120 127	36 40 45
2	Mallinckrodt "Parlodion," boiled 8 hrs. in 90 per cent alcohol (2 alcohol changes)	Very low	Very low	0.85	1.0	0.008	6.4	0.0013	108 110 114	28 18 22
3	Baker Collodion U.S.P., commercial preparation	0.4	0.2	1.1	1.5	0.03	5.9	0.004	115 121 130	52 40 60
4	Oxidized collodion; (Baker Collodion Cotton, "Pyroxilin") oxidized 48 hrs. with 1 M NaOBr and boiled several times with water	0.3	<0.2	3.0	3.3	0.29	4.1	0.26	130 140 158	145 138 178
5	Oxidized collodion (No. 4), washed 8 times with 95 per cent alcohol	<0.3	<0.2	2.7	2.9	0.19	4.5	0.11	135 144 158	180 145 172
6	Oxidized collodion (No. 4), washed 16 times with 95 per cent alcohol	<0.3	<0.2	2.6	2.8	0.03	5.7	0.0066	122 138 148	137 145 155
7	Oxidized collodion (No. 4, boiled 3 hrs. with 90 per cent alcohol	<0.3	<0.2	1.4	1.6	0.03	5.7	0.0066	135 140 150	125 134 120

a comparison between the acid number and the base exchange capacity of the various preparations on the one hand and their electrochemical activity on the other.

The *acid numbers* of the different preparations (column 6, Table I) vary only from 1.0 ml. of 0.01 N hydroxide per gm. for the most highly purified preparation to 3.3 ml. for highly oxidized collodion. Only a small fraction of the total acidity can possibly be due to semiesterified sulfuric acid.¹⁴ The ratio of the extremes of the observed acid numbers is only 1:3.3. The mean equivalent weights which correspond to these acid values are 100,000 and 30,000 respectively.¹⁵

¹⁴ On the basis of more indirect evidence we came in an earlier publication (Sollner, K., Abrams, I., and Carr, C. W., *J. Gen. Physiol.*, 1941, **24**, 467) to the conclusion that the sulfate content plays only a minor rôle if any in determining the electrochemical properties of collodion. This can now be shown definitely by comparing the sulfate contents (column 4 of Table I) with the corresponding acid numbers of column 6. If all the sulfate is present in the semiesterified form, a sulfate content of 0.1 mg. per gm. collodion corresponds fairly accurately to 0.1 ml. 0.01 N hydroxide solution. Thus, with the pure commercial preparations listed in Table I, the possible maximum contribution of sulfuric acid compounds to the overall acidity is only 8 to 13 per cent, with the oxidized collodion preparations less, corresponding to their higher titratable acidity.

¹⁵ The acid numbers of the commercial collodion preparations vary from 1.2 to 1.5 ml. of 0.01 N hydroxide solution per gm. collodion; this corresponds to (mean) equivalent weights of 67,000 to 83,000. We may assume that our collodion corresponds fairly closely to cellulose tetranitrate which carries two O—NO₂ groups per glucose residue and has a "molecular weight" of 252. Then, the mean chain length per carboxyl group would be 260 to 330 glucose residues. Using the same technique as with collodion, we found a similar chain length also with several acetylcellulose preparations. Our results are in good agreement with the corresponding values given by different authors for purified cellulose; Heymann and Rabinov (Heymann, E., and Rabinov, G., *J. Physic. Chem.*, 1941, **45**, 1152), *e.g.*, find 300, Sookne and Harris (Sookne, A. M., and Harris, M., *J. Research Nat. Bureau Standards*, 1940, **25**, 47; (*Nat. Bureau Standards, Research Paper RP 1313*)) about 600 glucose residues per carboxyl group. The carboxyl groups are believed to be present as the one end group of the cellulose molecule. (See further, *e.g.*, Sookne, A. M., Fugitt, C. H., and Steinhart, J., *J. Research Nat. Bureau Standards*, 1940, **25**, 61; (*Nat. Bureau Standards, Research Paper RP 1314*); Neale, S. M., and Stringfellow, W. A., *Tr. Faraday Soc.*, 1937, **33**, 881.) We may add that it is customary to assume on the basis of viscosity, osmotic, and ultracentrifuge studies, that the degree of polymerization of native cellulose and nitrocellulose derived therefrom is about 2,000 to 3,000 glucose residues per molecule. (See, *e.g.*, Ott, E., *Ind. and Eng. Chem.*, 1940, **32**, 1641.)

From the foregoing figures it is clear that the dissociable acidic groups occupy only a very small fraction of the surface of cellulose and nitrocellulose molecules.

In considering the *base exchange capacity* it is necessary for the reasons outlined above to consider the figures calculated on the basis of pH measurements (column 9). In the following discussion we will assume that the very low base exchange values found with some preparations can be taken at their face value. However, we must recall that these values, as discussed previously at length,⁴ are only maximum possible values; the figures given in the table for the cases 1, 2, and 3 are undoubtedly too high. The very great importance of the time factor will be discussed in the next section.

With the base exchange we find enormous differences between different preparations, less than 0.002 ml. of 0.01 N hydroxide solution per gm. for very inactive preparations (compare column 11), values as high as 0.26 ml. for oxidized collodion of high activity, and values down to 0.0066 ml. for oxidized collodion which was treated thoroughly with alcohol, but nevertheless has retained considerable electrochemical activity. The ratio of the lowest and the highest base exchange values reported in Table I is about 1:200, whereas the difference in the acid numbers of the same preparations is as noted above only 1:3.3.

We turn our attention next to the *correlation of acid number and base exchange capacity of the individual collodion preparations*. To do this, we compare the figures of columns 6 and 9 of Table I. This comparison shows that with the pure preparations 1, 2, and 3 not more than one in about 380 to 770 of all the acid groups is available for base exchange. With oxidized collodion, which was purified by repeated boiling with water, No. 4 of Table I, this ratio is about one in 13; with oxidized collodion, which was washed eight times with alcohol (No. 5), the ratio is increased to one in about 26; with more thoroughly purified oxidized collodion, the ratio increases to one in 240 for case 7 and one in 420 for case 6.

Thus we see that there are enormous differences in the availability for base exchange of the acidic groups of the various collodion preparations. The much higher base exchange capacity of oxidized collodion in the fibrous state is not so much due to its higher acid number as to its more open micellar structure. The variations between the several preparations are thus indicative of differences in micellar structure.

The more open structure of oxidized collodion is probably due to the presence of a small percentage of low molecular weight material which inhibits normal formation and arrangement of the micelles. That repelling forces between charged groups—the carboxyl groups—may play an important rôle in the formation of collodion micelles and their arrangement can on the basis of analogous cases be considered to be rather unlikely. The impurities which account for the open structure of oxidized collodion can be gradually removed by purification (cases 5 to 7 of Table I). But even very thorough purification yields a material of a somewhat more open structure (case 7) than the original unoxidized preparation, in spite of the fact that the acid number is reduced approximately to its original value.

We have so far not discussed the time factor with regard to the availability of the acidic groups for base exchange and the typical membrane functions. Our calculations so far have been made on the basis of the base exchange obtained after 48 hours. However, we must remember that membranes brought into a KCl solution assume (if they are not unduly thick) their final electrical

TABLE II

The Influence of the Time of Reaction on the Base Exchange of Various Collodion Preparations

A. Baker collodion U.S.P. (sample 3a)		
Time of reaction	pH values on treatment with 0.5 M KCl	Ml. 0.01 N NaOH per gm. dry collodion calculated from pH values
5 min.	—	—
30 min.	6.8	0.0005
1 hr.	6.4	0.0013
6 hrs.	6.2	0.0021
12 hrs.	6.0	0.0033
24 hrs.	5.9	0.0042
48 hrs.	5.9	0.0042
B. Oxidized collodion (sample 4a)		
5 min.	6.0	0.0033
30 min.	5.5	0.011
1 hr.	5.0	0.033
6 hrs.	4.6	0.084
12 hrs.	4.2	0.21
24 hrs.	4.1	0.26
48 hrs.	4.1	0.26
C. Oxidized collodion washed 16 times with 95 per cent alcohol (sample 6a)		
5 min.	6.5	0.0011
30 min.	6.3	0.0017
1 hr.	6.1	0.0026
6 hrs.	5.9	0.0042
12 hrs.	5.8	0.0053
24 hrs.	5.7	0.0066
48 hrs.	5.7	0.0066

properties; *i.e.*, conductance and membrane potential, within several minutes, as soon as the electrolyte has penetrated. Therefore only those groups which are placed so as to exchange readily can contribute significantly to the characteristic properties of membranes. Though it is impossible to estimate accurately the quantity of these readily exchanging groups, experiments on the influence of time on the base exchange will give some useful hints. As before, we consider the base exchange values which are calculated from the experi-

mental pH values. Table II contains such data. The preparations used (3a, 4a, 6a) are, as said before, very similar to Nos. 3, 4, and 6 respectively in Table I.

If we look at the base exchange values obtained after 5 or 30 minutes we certainly obtain a more correct picture of the availability of the dissociable groups which determine the typical electrochemical properties of collodion. In addition we must consider the fact that collodion fibers exhibit a greater number of dissociable groups in readily available places than more coherent and compact structures like most collodion membranes, particularly fairly dense ones. On this basis we come to the conclusion that the ratio of available dissociable groups in collodion membranes to those present in the preparation is hardly less than fifty times and probably rather a thousand times smaller than given above. We may therefore estimate this ratio to be rather 1:500 to 1:1,000,000 than 1:13 to 1:770, according to the preparation used.¹⁶

Finally we turn to the *correlation of acid number and base exchange capacity of the various collodion preparations to their electrochemical activity.*

The acid numbers (column 6, Table I) on which we base this comparison do not need any critical remarks. For base exchange values we use the data obtained in 48 hour exchange experiments (column 9, Table I), keeping in mind the above discussed restrictions of their significance.

As a measure of the electrochemical activity we use the mean of the three values given in column 11 of Table I for each of the collodion preparations. It is necessary to recall briefly the basis and significance of these figures.^{1, 2, 3} They represent the rate of anomalous osmosis observed under arbitrary but standardized conditions. Bag-shaped membranes (30 × 110 mm.) of approximately identical porosity (column 10 of Table I) filled with M/512 K₂SO₄ solution are immersed in water and the pressure rise in a capillary manometer tube is measured after 20 minutes. This pressure rise according to experiments by Loeb is for a given membrane proportional to the electrokinetic (ζ) potential times the membrane (ϵ) potential.¹⁷ Theoretical considerations of Sollner show that the relationship is more complicated.¹⁸ The pressure rise, that is the extent of the anomalous osmosis, is proportional to the electrokinetic (ζ) potential times the difference of the ϵ -potentials which arise between different parts or pores of the membrane; this difference is the driving force in the process of anomalous osmosis. The ζ -potential increases with increasing charge density

¹⁶ These ratios can be taken as a quantitative indication of the discrepancy between collodion membranes and membranes which behave as homogeneous "oil" phases. In the latter type of membranes all the functional groups (acid groups in our case) are available for the characteristic membrane function.

¹⁷ Loeb, J., *J. Gen. Physiol.*, 1922, **4**, 463; and many other papers in the preceding volumes of the same Journal.

¹⁸ Sollner, K., *Z. Elektrochem.*, 1930, **36**, 36; 1930, **36**, 234.

(number of exchanging groups per unit area) up to a certain limiting value which cannot be surpassed. Any further increase of the charge density does not lead to higher values of ζ . The correlation of driving force and charge density is not simple; it is not necessarily proportional to the measurable membrane potential. Higher charge density need not necessarily lead to an increase of the difference between local membrane or pore potentials. A simple consideration shows that a positive correlation cannot be expected with high charge densities. Thus the anomalous osmotic pressure rise is by no means a simple and straightforward quantitative measure of the "electrochemical activity," particularly with highly active preparations.

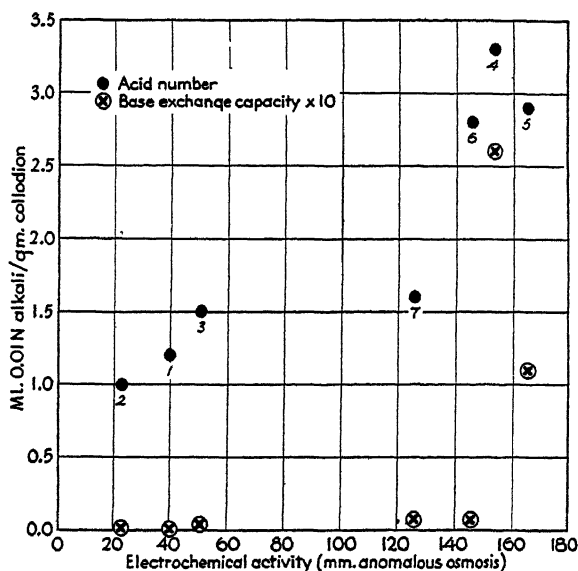


FIG. 3

With these reservations in mind we have plotted in Fig. 3 the electrochemical activity of the various preparations given in Table I against their acid numbers (dots) and base exchange capacities (crosses). In order to accommodate both sets of data in one graph the base exchange values are plotted enlarged ten times.

There is a reasonably good *correlation between acid number and electrochemical activity*. Low acid numbers are found with the preparations of lowest activity, the highest acid numbers are found with the most active preparations, though medium acid numbers are found with a fairly inactive (No. 3) as well as with a fairly active preparation (No. 7). An explanation of this latter discrepancy must be looked for in the above discussed difference in structure between the

commercial (No. 3) and the highly purified oxidized preparation (No. 7). The base exchange data indicate a somewhat more open structure of the latter material. One must assume that this results in a somewhat less smooth, more irregular, and rougher nature of the walls of the pores. This would lead to the exposure of a greater number of active groups in spots which can make an essential contribution towards the electrochemical activity.

The *correlation between base exchange capacity and activity* as pointed out in a previous paper is not as good. Low base exchange capacity is associated with low as well as with high electrochemical activity; high base exchange capacity is always associated with great electrochemical activity. Obviously the base exchange capacity which must be shown by a preparation to give rise to high electrochemical activity is very small, not more than barely measurable; any further increase in base exchange capacity does not cause a great positive increment in the electrochemical activity as measured by the anomalous osmosis method. This can be understood on the basis of two factors. First, as pointed out before, the electrochemical activity, as measured with the anomalous osmosis method, cannot be expected to increase steadily when the charge density increases over a certain level. The second factor must be looked for in this direction: the process of anomalous osmosis through the highly porous membranes used takes place essentially in the wider pores which traverse the membrane; it is inconsequential for this process if the functionally active pore walls have a microporous structure. If the pore walls have a structure of the latter type, parts of the membrane skeleton, which do not contribute to the process of anomalous osmosis, may easily be available for base exchange. Thus the more open microstructure, which as discussed above is found so pronouncedly with the less purified oxidized collodion preparations, easily may account for much of the apparent lack in parallelism between base exchange capacity and electrochemical activity as determined by the anomalous osmosis method. If the comparison between electrochemical activity and base exchange were based on short time base exchange values (Table II) a better agreement between the two sets of data would be found, since the differences in base exchange between the different preparations are less pronounced in short time experiments than after 48 hours. The inaccuracy of the very low short time base exchange values, however, makes it impractical to evaluate them quantitatively. In a forthcoming paper we intend to discuss certain aspects and consequences of the heteroporous character of collodion membranes.

One could attempt to use the acidity and base exchange capacity data, which were given above, for some comparison of collodion membranes with monomolecular films or for an estimation of the number of pores per unit area. However, we do not think that this at present would be a particularly fruitful beginning. The spread of the figures we could start out with is very wide; it also would be necessary to base the calculations on some additional estimates and some arbitrary assumptions. The problem of the number of pores per square

centimeter was treated in the past mostly on the basis of the assumption that pores of more or less uniform cross-section traverse the membrane in a fairly regular manner with little or no cross-connections. Such a situation, however, certainly does not prevail in dried collodion membranes or "porous" membranes of the type used in our previous work. We always must remember that the interstices in a collodion membrane are irregular, non-uniform, and inter-connected; furthermore many dead-end spaces exist undoubtedly.

The availability of the pore space for the most typical membrane function, the penetration of third substances, is moreover not only governed by the complex and unknown interplay of the above mentioned and similar factors, but also by the characteristics of the third substance. Pathways which are available for small molecules are unavailable for bigger ones.

In order to characterize structures like membranes with regard to their important functional properties, it is necessary to look for new approaches which are less formal than those ordinarily used in the past. A study of the functional properties of the membranes, their permeability, their ionic selectivity, *i.e.* their electromotive properties, and their conductance in solutions of various electrolytes and different concentration will furnish the data of most interest. Knowledge of the geometrical structure of membranes is of interest mainly in as far as it helps to coordinate, to explain, and to visualize the functional membrane properties.

This does not mean that the application of other physical methods in elucidating the structure of collodion membranes is of no interest. Quite to the contrary, we think that such methods should be exploited to the limit; such studies¹⁹ are bound to give us a fuller understanding of the data which may be obtained with the above mentioned methods of functional membrane characterization.

Our next problems will be to prepare fairly uniform membranes with well

¹⁹ Due to the wartime conditions we shall not be able to attack the purely physical aspects of our wider problem in the near future. The obvious approach would be the use of x-rays to obtain some information as to the size and arrangement of the micelles in collodion fibers, in porous and in dried membranes. We also think that the electron microscope could conceivably be of great help in the study of extremely thin membranes. If, *e.g.*, the molecules are arranged into micelles in such a manner that many end carboxyl groups come to lie next to each other, like the points of a bundle of pencils, then these groups, if neutralized with ions of a metal having a high atomic number (*e.g.*, Pb or Hg), may become visible with the electron microscope.

Another problem that requires fundamental investigation is the coordination of our findings with regard to the scarcity of dissociable groups in collodion with the conventional concepts of the electric double layer and of the electrokinetic potential. A combination of electroosmotic and cataphoretic studies with collodion should go far towards elucidation of this question. Some of the apparent difficulties will be resolved, at least partially, by the assumption of structural irregularities which we hope to discuss at greater length in a forthcoming paper.

defined electrochemical characteristics, and to investigate their electrochemical properties; *i.e.*, their conductance and electromotive behavior in solutions of different electrolytes.

For much valuable help we are indebted to Dr. Charles W. Carr.

SUMMARY

1. The electrochemical behavior ("activity") of collodion membranes depends upon acidic, dissociable groups located in the interstices of the membranes. The active groups can be determined by base exchange measurements. High base exchange capacity is always found with preparations of great "electrochemical activity;" medium and low base exchange capacities occur with electrochemically active as well as with inactive preparations. The observed base exchange capacity is determined by two factors: the inherent acidity of the collodion (its mean equivalent weight) and the submicroscopic micellar structure of the collodion. A comparison of the base exchange capacity of various collodion preparations and their inherent acidities therefore allows certain conclusions to be drawn concerning the relative availability of the micellar surfaces in the different preparations.

2. The inherent acidity of various collodion preparations, their "acid number," was determined by electrometric titration. Collodion in the acidic state, *i.e.* after exchange of all other cations for H^+ ions, was titrated in an organic solvent mixture with alcoholic KOH using a quinhydrone electrode. Details of the experimental procedure are given in the paper. The acid numbers, expressed in milliliters of 0.01 *N* KOH per gram dry collodion, vary from 1.0 for a highly purified collodion preparation of very low electrochemical activity to 3.3 for a highly oxidized sample of very high activity. Acid numbers of about 1.5 (corresponding to an equivalent weight of about 67,000) are found both with inactive commercial and with fairly active oxidized preparations. The base exchange capacity of the same preparations in the fibrous state as measured after 48 hours of exchange time varies from 0.0013 ml. 0.01 *N* NaOH per gm. dry collodion for the most inactive preparation up to 0.26 ml. 0.01 *N* NaOH per gm. for the most active preparation. Thus the acid numbers over the whole range investigated differ only in the ratio of 1:3.3, whereas the base exchange values differ in the range of 1:200.

3. In the inactive preparation only one in 770 acid groups is available for base exchange, in the most active collodion one group in 13; values between these extremes are found with commercial and alcohol purified oxidized preparations.

4. The high base exchange capacity of the electrochemically active preparations is not so much due to their higher acid number as to their more open structure. This difference in structure is ascribed to the presence of a small

fraction of low molecular weight material which inhibits normal formation and arrangement of the micelles.

5. Short time base exchange experiments with fibrous collodion indicate that the number of acid groups available for the typical electrochemical membrane functions may be estimated to be about 50 to 1000 times less numerous than those found in the 48 hour base exchange experiments. It is estimated that in membranes prepared even from the most active collodion not more than one in 500 acid groups may be available for the typical membrane functions; with the less active preparations this ratio is estimated to be as high as one in 1,000,000 or more.

THE STRUCTURE OF THE COLLODION MEMBRANE AND ITS ELECTRICAL BEHAVIOR

IX. WATER UPTAKE AND SWELLING OF COLLODION MEMBRANES IN AQUEOUS SOLUTIONS OF ORGANIC ELECTROLYTES AND NON-ELECTROLYTES

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I

In a recent publication Carr and Sollner¹ have reported on the water uptake and the swelling of collodion membranes in water and solutions of strong inorganic electrolytes. They determined the weight and volume changes of collodion membranes when placed in water and when transferred from water to solutions of strong electrolytes. It was found that dried collodion membranes swell reversibly to an appreciable extent when placed in water, the swelling varying from 5 to 11 per cent depending on the brand of collodion. The water uptake as determined by the weight increase is larger than could be accounted for by the volume increase, swelling accounting for only 60 to 70 per cent of the water taken up by the membranes. When the "water-wetted dried" collodion membranes were transferred from water to solutions of various strong electrolytes, there was no detectable change in volume. Similarly, when "porous" membranes were transferred from water to solutions of strong electrolytes, there was no significant volume change.

Without giving further details, Carr and Sollner¹ stated that the problem of water uptake and swelling of collodion membranes is more complex in aqueous solutions of many organic compounds. This is of interest in view of the fact that the permeability of collodion membranes to various organic substances in aqueous solutions was found by several investigators to be anomalously high.

Michaelis and Fujita² reported that strychnine, quinine, and other alkaloid cations penetrate dried collodion membranes with approximately the same ease as do sodium ions, which undoubtedly are much smaller.

Collander,³ measuring the relative permeability of about thirty organic non-electrolytes and weakly dissociated compounds, mostly acids, found that with a few exceptions the permeability decreased regularly with increasing molecular

¹ Carr, C. W., and Sollner, K., *J. Gen. Physiol.*, 1943, **27**, 77.

² Michaelis, L., and Fujita, A., *Biochem. Z.*, Berlin, 1925, **161**, 47.

³ Collander, R., *Soc. Scient. Fennica, Commentationes Biol.*, II. 6., Helsingfors, 1926.

size. The compounds which in his experiments deviated most strongly from this regular pattern are propionic acid, valeric acid, monochloroacetic acid, phenol, and *m*-nitrophenol. It is important to note that a membrane kept for some time in a solution of *m*-nitrophenol was found by Collander to contain much more of the solute than would be expected if its concentration in the water contained within the membrane were the same as in the outside solution. Collander links the unusually high permeability of the *m*-nitrophenol with this effect.

Höber⁴ determined the relative permeability through positive membranes of a number of organic and inorganic anions in solutions of their Na salts. For this purpose he used collodion membranes impregnated with a basic dye-stuff. It would be premature to discuss Höber's very interesting results in detail here. It must suffice to state that in many cases the permeability of the organic anions is much higher than the permeability of inorganic anions of smaller molecular size. *E.g.*, the fatty acid ions from acetate to the valerate ion possess permeabilities comparable to that of the much smaller chloride ion; and the fatty acid anions above valeric acid have even greater permeabilities. This behavior Höber attributes to the surface activity of the organic ions which are believed to be concentrated on the pore walls, and thus to move faster across the membrane.

In view of the fact that air-dried collodion membranes swell in water,¹ it seemed advisable to investigate whether the solutions of the substances that were found to exhibit anomalous permeabilities might have a specific swelling effect. The detection of such an effect, of course, would be most important in determining the limits of applicability of collodion membranes to permeability studies; in addition it would cast light on the findings of the above mentioned investigators. We therefore have investigated the swelling of collodion membranes in aqueous solutions of some organic substances and the uptake of solute from these solutions by the membranes.

The organic substances for this investigation were chosen preferentially from three classes of compounds. One group was selected from among those weakly or non-dissociated substances used by Collander which would be most likely to influence the swelling of collodion, such as fatty acids, phenol, and nitrophenol; secondly, some definitely hydrophilic compounds such as glycerine, glucose, and citric acid; and thirdly some strong organic acids, sulfonic acids, were tested, the sodium salts of which were found by Höber to behave very anomalously.

This study has been confined to dried collodion membranes since any specific swelling effect that may be found with dried collodion membranes would also result in a change of structure of "porous" membranes.

⁴ Höber, R., *J. Cell. and Comp. Physiol.*, 1936, 7, 367.

II

The experimental procedure is briefly as follows: flat dried collodion membranes were prepared, their weights determined, and their volumes ascertained with a pycnometer filled with mercury; they then were placed into the solutions of the various organic compounds. After measured duration of immersion in the solutions, the membranes were removed, blotted, and their weights and volumes again determined. Finally, in a number of representative cases the amount of solute contained in the membranes at equilibrium with various solutions was determined.

The technique of the weight and volume measurements was the same as used by Carr and Sollner which was described in another paper.¹

In some cases the weight and volume measurements were made at relatively frequent intervals in order to ascertain the swelling of the membranes as a function of the time. In all cases enough determinations were made to assure that equilibrium values of weight and volume had been reached. The material used throughout this investigation was Baker U.S.P. collodion.

To determine the amount of organic solute contained in the membranes, the following procedure was used: the membrane was removed from the solution and momentarily dipped into distilled water; then it was placed into a 150 × 15 mm. test tube containing *ca.* 10 ml. of distilled water which covered it completely. After a sufficient amount of the organic substance had diffused out of the membrane, in most cases after a few days, the membrane was removed from the solution and the latter was titrated. The membrane was placed again in distilled water and the resulting solution was titrated as before after several days. This procedure was repeated until successive titrations yielded zero values. A membrane which was free of organic solute was used as a control and titrated along with the others. With the acids the titration was carried out with 0.02 N NaOH, using a syringe micro buret which allowed readings to 0.002 ml. The total amount of reagent required for the control was subtracted from the amount required for the titration of the membranes. The phenol solutions were titrated in a similar manner with 0.1 N solution of KBr-KBrO₃. In all cases the analyses were carried out after swelling equilibrium had been established.

The results of the weight and volume measurements are given—as by Carr and Sollner—as milligrams and cubic millimeters weight and volume increase respectively per cubic centimeter of dry membrane, which occur when dry collodion membranes are immersed in the various solutions.

The accumulation of solute by the membranes was calculated as follows: the amount of substance actually present in a membrane was divided by the quantity that would be present if the concentration of the solute in the solution which is contained within the membrane were the same as in the bulk of the

solution. This ratio obviously is a quantitative measure of the relative accumulation of solute within the membrane.

The quantity of solute present in the membrane is found by analysis. The quantity of solute that would have entered the membrane if the measurable weight increase were caused by the uptake of unchanged solution can readily be calculated from the weight increase and the known specific gravity and the concentration of the solution. The former quantity divided by the latter gives the desired ratio which we call the "accumulation factor."

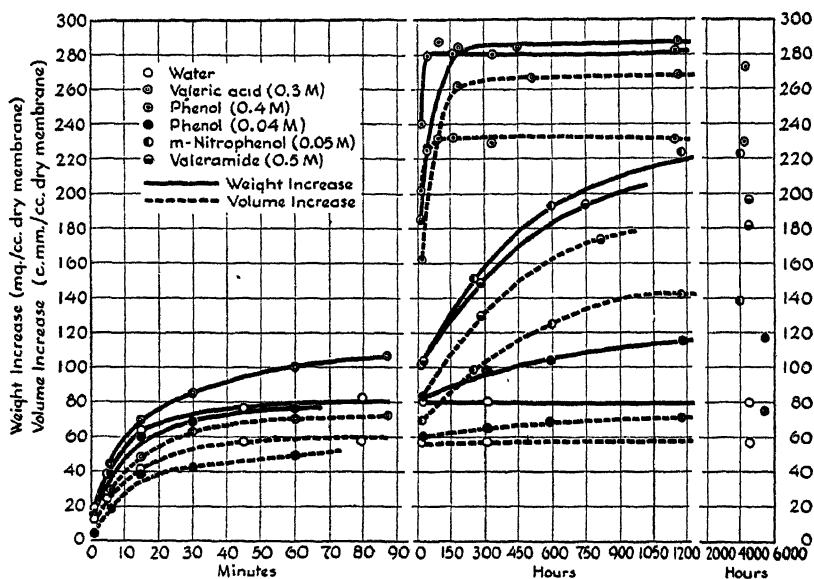


FIG. 1

It is of interest to compare the quantity of solute contained in the membrane with the corresponding weight or volume increases. The clearest picture of this relationship is obtained by comparing the quantity of solute taken up per cubic centimeter of dry membrane with the corresponding "characteristic additional weight increase." This latter quantity we define as the weight increase in solution minus the weight increase in water per cubic centimeter of dry collodion.

III

Fig. 1 gives the relative volume and weight increases of dried collodion membranes in aqueous solutions of several organic compounds as a function of time. The equilibrium values of the weight and volume increases per cubic centimeter

TABLE I

The Interaction of Dried Collodion Membranes with Aqueous Solutions of Various Organic Compounds

1	2	3	4	5	6	7
Solute	Concentration of solution	Weight increase per cc. dry membrane	Volume increase per cc. dry membrane	"Accumulation factor"	Uptake of solute per cc. dry membrane	"Characteristic additional weight increase" per cc. dry membrane
	<i>mols/liter</i>	<i>mg.</i>	<i>c.mm.</i>		<i>mg.</i>	<i>mg.</i>
Water.....	—	80	57	—	—	—
Acetic acid.....	0.54*	97	72	8.7	25.4	17
Propionic acid.....	0.52*	114	90	13.0	52.9	34
Butyric acid.....	0.28*	125	101	22.7	67.8	45
Butyric acid.....	0.49*	161	137	15.6	108	81
Valeric acid.....	0.26*	276	260	32.1	232	196
Monochloroacetic acid.....	0.53*	152	103	9.3	67.8	72
Ethyl alcohol.....	0.5	83	57	§	§	3
Isobutyl alcohol.....	0.5	122	108	§	§	42
Isoamyl alcohol.....	0.5	136	127	§	§	56
Formamide.....	0.5	85	66	§	§	5
Valeramide.....	0.5	196	181	§	§	116
Phenol.....	0.042*	117	76	114	47.4	37
Phenol.....	0.38*	282	232	20.8	234	202
<i>m</i> -Nitrophenol.....	0.05	224	145	§	§	144
Benzene sulfonic acid.....	0.5	75	56	0.00	0.0	-5
β -Naphthalene sulfonic acid.....	0.5	78	57	0.00	0.0	-2
Glycerine.....	0.5	96†	72†	§	§	6
Glucose.....	0.5	82†	70†	§	§	-8
Citric acid.....	0.5	99†	75†	0.00	0.0	9

* Concentration determined analytically after establishment of swelling equilibrium.

† These membranes were cast at a later date—the weight and volume increases in water for the membranes in this group are: 90 mg. per cc. dry membrane, and 70 c.mm. per cc. dry membrane respectively.

§ Not analyzed.

of dry membrane for all the substances tested are summarized in columns 3 and 4 of Table I. The "accumulation factors" for several solutes are given in column 5; the solute uptake in milligrams per cubic centimeter of dry mem-

brane and the "characteristic additional weight increase" in milligrams per cubic centimeter of dry membrane are shown in columns 6 and 7.

IV

The data presented in columns 3 and 4 of Table I show that the dried collodion membrane exhibits widely different behavior when placed in solutions of various organic compounds; the weight and volume increases of the membrane differ greatly with the nature and concentration of the solute, being in many cases far larger than in water.

No significant specific swelling effect is observed with the 0.5 M solutions of ethyl alcohol, benzene sulfonic acid, β -naphthalene sulfonic acid, glycerine, glucose, and citric acid. The weight and volume increases are significantly larger than in water alone with acetic acid and the higher fatty acids, monochloroacetic acid, isobutyl and isoamyl alcohol, valeramide, phenol, and *m*-nitrophenol. With formamide the results seem to be ambiguous. In the homologous series of the fatty acids, alcohols, and amides the swelling effect increases with increasing molecular weight. In view of this, one may be inclined to think that a real though small specific swelling effect exists in the case of the formamide solution. The swelling action of a solute is greater, the higher its concentration.

Fig. 1 shows for a few representative cases that the rate at which swelling equilibrium is attained differs markedly for the various solutions. When a dried membrane (prepared from Baker collodion U.S.P.) of about 0.15 mm. thickness is placed in water, swelling equilibrium is reached in approximately 80 minutes; a similar behavior is observed in the case of the solutions of those substances which do not show any specific swelling effect. With solutions which show a pronounced swelling effect this time varies from about 100 hours for 0.26 M valeric acid and 0.38 M phenol to about 1000 hours with 0.042 M phenol and 0.05 M *m*-nitrophenol solutions.

From the data of column 5 it is seen that the relative accumulation of the organic solute within the membrane, expressed by the "accumulation factor," differs very widely with the various solutes. In the two instances in which two concentrations of the same solute were tested the "accumulation factor" is higher with the more dilute solution.

A comparison of columns 3 and 4 with column 5 shows a very marked parallelism between the swelling caused by a solute and its tendency to accumulate within the membrane; the compounds which cause the most pronounced swelling are also accumulated most strongly. The increase in the "accumulation factor" with increasing molecular weight in the homologous series of the fatty acids may be specially mentioned. The substances which do not show any specific swelling effect show "accumulation factors" smaller than one; this means that the water in the membrane contains less of the solute than it does

in the surrounding solution. Three of the organic substances tested, benzene-sulfonic acid, β -naphthalene sulfonic acid, and citric acid, are actually excluded completely from the membranes.⁵

To use conventional terminology, one may say that typical "hydrophilic" substances have little influence on the swelling of collodion membranes and are not accumulated in them, whereas "carbophilic" substances make the membranes swell and are accumulated.

The characteristic swelling which is observed with some solutes is entirely due to the accumulation of a corresponding quantity of the solute within the membrane, as can be seen from a comparison of columns 6 and 7. The absolute uptake of solute per gram of dry membrane (column 6) is at least as great as the "characteristic additional weight increase" per gram of dry membrane in the same solutions (column 7); with some solutes it seems to be significantly greater. The water content of the membranes in equilibrium with the solutions is therefore scarcely as high, in some cases probably significantly lower, than in pure water.⁶ A collodion membrane which has taken up a significant quantity of organic solute must be considered as a structure substantially different from a similar membrane wetted with water.

A pronounced parallelism is apparent between the anomalously high permeabilities described by Collander with certain compounds and their accumulation and the swelling effect caused by their solutions. All the compounds Collander found to deviate from the expected permeability—phenol, *m*-nitrophenol, propionic acid, monochloroacetic acid, and valeric acid—exhibit a pronounced swelling effect and are accumulated in the membranes. Substances which in our experiments do not show a specific swelling effect and are not accumulated—or which on the basis of their structure must be expected to act so—show normal permeability characteristics according to their molecular size.⁷

⁵ The "accumulation factor" was also determined for two inorganic electrolytes, HCl and HNO₃; both show an accumulation factor smaller than 1; 0.03 in the case of HCl and 0.78 in the case of HNO₃. It is of interest to note that HNO₃, which, as shown by Collander,³ penetrates collodion much faster than does HCl, shows a much higher accumulation factor than the latter. Nitric acid is known to have a strong affinity to cellulose, forming at higher concentrations a definite molecular compound (Knecht's compound).

⁶ The unusually large differences between the figures for the weight and volume increases in the cases of monochloroacetic acid, phenol, and nitrophenol are of course due to the high specific gravities (1.58, 1.07, and 1.48 respectively) of these substances. The converse holds true for the unusually small differences between the values for weight and volume increase found with valeric acid (sp. gr. 0.94) and isoamyl alcohol (sp. gr. 0.81).

⁷ Collander did not test the sulfonic acids. No data seem to be available on their permeability through collodion membranes.

With positive, dyestuff-impregnated collodion membranes the parallelism between anomalous permeability and accumulation is not complete. The unusually high permeability of fatty acid ions in neutral solution is paralleled by the swelling effect and the accumulation of the free acids. The same is not true, however, for the high permeability of aromatic sulfonic acid anions in neutral solution. This discrepancy is hardly surprising in view of the different conditions in Höber's permeability and our swelling and accumulation studies with negative membranes.

On the basis of available information it is impossible to discuss in a satisfactory manner the molecular mechanisms causing the accumulation and the swelling effects and the correlated increase in permeability. The situation is undoubtedly fairly complex. Accumulation can result from adsorption on the micellar surfaces, from filling of intermicellar spaces, and from an absorption of the solute by the micelles. Roughly corresponding to these three degrees of interaction between solute and membrane one would expect little or no specific swelling, moderate specific swelling, and strong specific swelling which in its highest degree approaches dissolution.

These processes obviously are not independent of each other; the observed effects depend upon the nature of the solute and its concentration as well as of the time of interaction between solution and membrane. At low concentrations surface adsorption must be favored over absorption in the interior of the collodion micelles. In a similar manner the alkali salts of organic acids may be strongly adsorbed, but they hardly would dissolve in the mass of the collodion, though the weakly dissociated free acids, which are essentially non-electrolytes, may readily do so.

That the permeability of the accumulated solutes is greater than can be expected on the basis of their molecular size undoubtedly can be due to several different mechanisms. There is first the possibility that a solute which is concentrated in the adsorption layer on the micellar surfaces migrates in the adsorbed state,⁸ as suggested by Sollner⁹ and Höber.⁴ If the adsorption is more pronounced, liquid layers of the solute wetting the membrane may result which, stretching continuously between the two sides of the membrane, could easily account for an increased rate of penetration of the solute. If the micelles absorb and dissolve the solute there is further the possibility that some of the solute diffuses through the swelled collodion micelles themselves. Swelling, furthermore, is bound to result in some change in the pore structure of the membrane which may also lead to an increased rate of permeation of the solute. That the time factor too must be considered is obvious. Adsorption is a fast process and its consequences therefore must be apparent in short time experi-

⁸ Volmer, M., and Estermann, I., *Z. Physik*, 1921, 7, 13. Volmer, M., and Adhikari, G., *Z. Physik*, 1925, 35, 170.

⁹ Sollner, K., *Kolloid-Z.*, 1933, 62, 31.

ments; absorption and dissolution are relatively slow processes; their full effect is only felt after prolonged contact between solution and membrane.

From the foregoing discussion it is clear that swelling, accumulation, and anomalous permeability need not necessarily be parallel in all cases. One can readily conceive of instances in which accumulation without swelling may cause an increased permeability.¹⁰

The interaction of a first solute with the membrane must affect the permeability to second solutes. Blocking of some pathways by the first solute,¹¹ changes in the geometrical and electrical structure of the membrane, and interaction between the second solute and the first which is present locally in high concentration, as well as other effects, can occur, depending on the nature and concentration of the solutes. This makes general predictions hazardous. Careful experimental investigation alone can clarify this problem.

Further discussion seems superfluous since the purpose of the present investigation is only to demarcate the approximate limits of the usefulness of the collodion membrane as a general model of the non-swelling, inert, porous membrane.

Collodion membranes seemingly act as inert membranes with the aqueous solutions of the strong, weakly adsorbable¹² electrolytes and those typically hydrophilic organic substances which are reasonably free from carbophilic groups. With all other substances the situation needs careful individual examination.

The instances of a specific interaction between organic solutes and collodion membranes are apparently much more numerous than has previously been assumed. The anomalies of permeability resulting from this interaction which were observed in the past with certain carbophilic compounds are now more clearly understood as a result of the specific interaction of solute and membrane.

In future work it therefore will be necessary to ascertain the extent of a possible interaction between any given solute and the membrane. A sharp distinction must be made between phenomena which are characteristic for porous membranes in general and observations which are due to a specific interaction between solute and membrane.

The use of membranes prepared from other material than collodion, *e.g.*, denitrified collodion (cellulose) or various silicates obviously will be helpful

¹⁰ Membranes of porous character have interstices of a whole spectrum of sizes. If on account of its molecular size the solute cannot enter the smaller pores but may enter the bigger ones and be adsorbed there, then it is possible that in spite of an overall "accumulation factor" smaller than 1, an abnormally high permeability due to adsorption may be found.

¹¹ Weech, A. A., and Michaelis, L., *J. Gen. Physiol.*, 1928, **12**, 55.

¹² Nitric acid according to the results of Collander and ourselves cannot be included in this group.

in clarifying the question of the effects of a specific solute-membrane interaction.

The interaction between solute and membrane is, of course, a phenomenon of considerable interest in its own right, primarily since such interaction undoubtedly plays an important rôle in living membranes. For an investigation of this problem, however, it may be advisable to use membranes more readily comparable to living tissues than collodion.

SUMMARY

1. Dried collodion membranes are known to swell in water and to the same limited extent also in solutions of strong inorganic electrolytes (Carr and Sollner). The present investigation shows that in solutions of organic electrolytes and non-electrolytes, the swelling of dried collodion membranes is not as uniform, but depends on the nature of the solute.

2. The solutions of typically "hydrophilic" substances, *e.g.*, glycerine, glucose, and citric acid, swell collodion membranes only to the same extent as water and solutions of strong electrolytes.

In solutions of typically carbophilic substances (*e.g.*, butyric acid, valeric acid, isobutyl alcohol, valeramide, phenol, and *m*-nitrophenol) the swelling of the membranes is much stronger than in water, according to the concentration used. For the brand of collodion used the swelling in 0.5 M solution was in some cases as high as 26 per cent of the original volume, as compared to 6 to 7 per cent in water. Therefore, in these solutions the "water-wetted dried" collodion membrane is not rigid, inert, and non-swelling, but behaves as a swelling membrane.

3. The solutes which cause an increased swelling of the membranes are accumulated in the latter, the degree of accumulation being markedly parallel with the degree of their specific swelling action.

4. The anomalously high permeabilities of certain carbophilic organic solutes reported by Michaelis, Collander, and Höber find an explanation in the specific interaction of these substances with collodion.

5. The use of the collodion membrane as a model of the ideal porous membrane is restricted to those instances in which no specific interaction occurs between the solute and the collodion.

THE EFFECT OF URETHANE ON THE CONSUMPTION OF OXYGEN AND THE RATE OF CELL DIVISION IN THE CILIAE TETRAHYMENA GELEII

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INTRODUCTION

The effects of narcotics on several types of cell including yeast, luminous bacteria, and brain cortex have suggested to us (Fisher and Stern, 1942) that the normal oxygen consumption of those cells may be due to two or three parallel chains of oxygen-consuming reactions. It was shown in yeast that normal cell division appears to depend upon the operation of a specific one of these fractions of the total oxygen consumption of the cell. Implications having a possible general interest arise from this interpretation of the effects of narcotics (Fisher, 1942). It is consequently desirable that its validity be thoroughly explored.

Within the past few years the conditions necessary to maintain bacteria-free cultures of many protozoa have been determined. It is therefore feasible to study the relation between cell respiration and cell division in these cells in the manner applied to yeast. Experiments have now been made for this purpose on the ciliate, *Tetrahymena geleii* (Ferguson, 1940), as many of the conditions and characteristics of growth in it are known, and since it was already being maintained in pure culture for other purposes by one of us (Ormsbee, 1942). Specifically, the effects have been determined of a series of concentrations of ethyl carbamate (urethane) on the rate of cell division and on the rate of oxygen consumption in that organism.

Material and Methods

The Hetherington strain of the ciliate, *Tetrahymena geleii* was used in the experiments to be reported. The cultures were grown in 250 ml. flasks of the type described by Kidder (1941). Each flask contained 100 ml. of the medium, a 2 per cent solution of Difco proteose peptone in redistilled water prepared in a Pyrex still. The methods of making sterility checks and population counts are described by Ormsbee (1942). To determine the effect of urethane on growth, various quantities of this substance in solution (sterilized by Seitz filtration) were added to the cultures after these had reached the exponential phase of growth.

The organisms used for the respiratory measurements were taken from flasks for which the population curve was determined as growth proceeded. Experiments were made on cells from cultures in the exponential phase of growth as well as from cul-

tures which had reached the stationary phase. To obtain material for use in the respirometers the ciliates were separated from the medium by centrifugation at 1500 R.P.M. and then washed three times with 0.005 M phosphate buffer at pH 6.9. Oxygen consumption was measured in that substrate-free buffer and also in the nutrient proteose peptone solution using Warburg's direct method (Dixon, 1934). The rate of shaking was not a limiting factor at the density of the suspension of organisms (100,000 to 500,000 organisms per ml.) used for the measurements.

RESULTS

Each respiratory experiment typically lasted 1.5 to 2.5 hours and the rate of oxygen consumption during this interval was constant even in the presence of urethane.

TABLE I

The Average Oxygen Consumption of Tetrahymena geleii at Different Concentrations of Ethyl Carbamate

Ur concentration	No. of experiments	Average respiration as per cent control (= U)	Average inhibition as per cent control (= I)	$\frac{U}{I}$	$\log 10^2 \frac{U}{I}$
μ					
0.023	14	82	18	4.56	2.66
0.045	15	80	20	4.00	2.60
0.090	16	75	25	3.00	2.48
0.12	5	72	28	2.57	2.41
0.14	18	65	35	1.87	2.27
0.17	14	57	43	1.33	2.12
0.20	14	47	53	0.89	1.95
0.31	4	36	64	0.56	1.75
0.38	3	9	91	0.10	1.00

The data from which Fig. 1 was prepared.

Observations made with the organisms suspended in buffer containing various concentrations of the narcotic are given in Table I and Fig. 1. The data are plotted on the axes most conveniently used to test the possibility of describing the observations by an equation derived from the mass law (for details see Fisher and Stern, 1942). It is evident that the points do not fall along a straight line. Actually the bend in the curve of the figure is so sharp that two straight lines meeting at a urethane concentration of 0.1 M ($\log 10^2 [\text{Ur}] = 1.0$) would describe these points quite accurately. These data closely resemble the observations on yeast.

The concentrations of urethane quoted in the figure were calculated from the quantities added to the vessels. This procedure is permissible since the degree of inhibition under the conditions prevailing in these experiments was found

to be independent of the number of organisms present. Hence the calculated concentration was not appreciably altered by the quantity of narcotic taken up by the cells.

Observations made on the viability of the protozoan after treatment with the narcotic lead to the conclusion that the inflexion shown in the figure must be considered a characteristic of normal cells. Complete removal of the narcotic effects seems to be possible after exposures to 0.31 M solutions for one-half hour. Reversibility was demonstrated by following the growth rate of a culture inoculated with organisms which had been treated with urethane. In

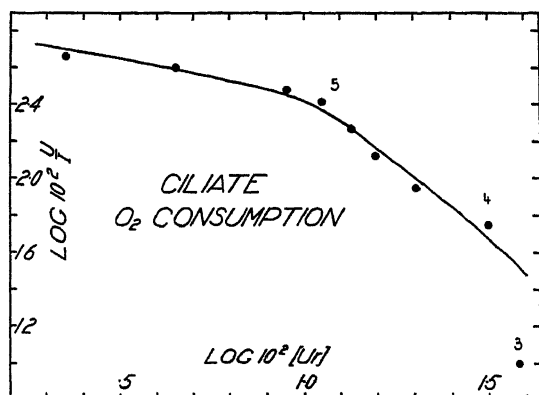


FIG. 1. The effect of ethyl carbamate on the oxygen consumption of *Tetrahymena geleii*. U is the rate of oxygen consumption in the presence of a given molar concentration of the inhibitor Ur . I is the difference between U and the normal respiration in the absence of the inhibitor. The notation beside three of the points indicates the number of separate observations averaged to obtain each of them. Each remaining point is the average of fourteen to sixteen individual measurements. Appreciable cytolysis occurs at the highest value of Ur used and that point therefore should be given less weight than the other points.

addition the motility and gross appearance of the cells return to normal within 40 minutes after the organisms are removed from the urethane solution into distilled water or proteose peptone. Cytolysis of the organisms is produced by 0.38 M urethane nearly instantly, but it is clear that the bend in the curve shown in Fig. 1 occurs at a concentration of the narcotic, approximately 0.1 M, well below that which causes cell damage. As in the case of yeast, one must assume that the particular relation found between concentration and effect, and shown in Fig. 1, is a property of the cellular respiratory systems. Since it is not a straight line it must be concluded, unless we imagine the existence of unrecognized complications, that the narcotic does not combine quantitatively in accordance with the mass law at a single locus in the cell. It is also unlikely that the observed effect is due to the action of the narcotic at a large number of

different catalysts in the cell, for in this case the average result would undoubtedly be similar to that characteristic of the single locus.

It was conceivable that the effect of the narcotic might be related to the particular growth phase of the population from which the cells were taken and perhaps also to the presence or absence of nutrient materials. Both of these factors exert a pronounced effect on the absolute rate of oxygen consumption by this ciliate (Ormsbee, 1942). We found that the growth phase of the culture does not affect significantly the curve which results when the data are plotted

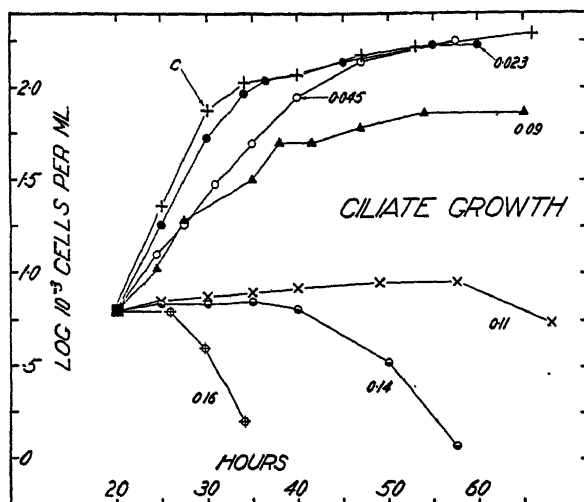


FIG. 2. The effect of urethane on the rate of cell division in *Tetrahymena geleii*. Each curve represents the number of cells present in the culture at various times. Urethane was added to give the molar concentrations indicated beside each curve when the cultures were 20 hours old. Curve C represents growth in a control culture without added narcotic.

as in Fig. 1. The values shown in that figure actually include measurements on cells from cultures in the exponential phase of growth as well as from cultures which had reached the period of stationary growth. Proteose peptone increases the rate of oxygen consumption by these cells three to five times and we have noted that the respiration in the presence of this nutrient may be slightly more sensitive to urethane than it is without it. Preliminary experiments showed that the shape of the curve relating inhibitor concentration and rate of oxygen consumption, plotted as in Fig. 1, is not significantly altered by the presence of nutrient. Consequently those observations were not extended.

Portions of the growth curves for *Tetrahymena geleii* in various concentrations of urethane are shown in Fig. 2. These data represent eight experiments including forty-four separate cultures. Each point, as in the case of the respira-

tory data, is the average of several values. It will be noted that the degree of inhibition increases as the concentration of urethane rises, until at approximately 0.1 M, cell division is inhibited completely.

Fig. 2 also shows that death due to urethane occurs to an appreciable degree in a 0.16 M solution only after an exposure of 5 hours.

It is apparent that the minimum concentration of narcotic which produces a complete block of cell division is the concentration at which the bend in the plotted respiratory data (Fig. 1) occurs. That concentration removes only 26 per cent of the oxygen consumption. It is at once evident that one series of chemical reactions cannot be responsible for both oxygen consumption and cell division, for a given chain of reactions cannot be inhibited only 26 per cent and at the same time 100 per cent. The unequal effects of urethane on oxygen consumption and cell division constitute then specific evidence for the existence of a system of reactions, the "activity" system, concerned with growth and cell division which is distinct from other reactions responsible for oxygen consumption in the cell. The activity system itself must be connected with oxygen consumption in some way, however, because cell division in this ciliate depends upon the presence of oxygen (Phelps, 1935) as it does of course in all aerobes.

It is not possible to consider at this time the several conceivable mechanisms by which the consumption of oxygen might be related to the function of the activity system (*cf.* Fisher, 1942). However, one of these provides a satisfactory description of the data in considerable detail and is therefore worthy of attention. From the fact that cell division in this organism is dependent on oxygen, it is reasonable to infer that the activity system consumes oxygen so that there must be two systems of oxygen-consuming reactions in *Tetrahymena geleii*. Suppose that in each there is an enzyme reversibly inhibited by urethane. For the reaction of the narcotic (N) with the enzyme (E) we can, with respect to each site of narcotic action write



the constant a representing the number of units of narcotic which combine with one unit of enzyme in order to form the inactive complex (EN_a). If the mass law governs the reaction of the inhibitor at each of the two sites, then an equation can be set up to describe the relation between concentration and inhibition of oxygen consumption for each of the two systems respectively. From the two equations the total oxygen consumption (= sum of the consumptions by the two systems) remaining at each narcotic concentration and the total oxygen consumption lost due to the inhibitor can be calculated. These calculated values of U and I respectively for the whole cell may then be treated as were the experimental data in Fig. 1. The line in that figure was obtained in this way from the curves shown in Fig. 3. It is apparent that the form of the calculated relation closely approximates that of the observations.

It should be noted that the constants used in the two equations cannot be calculated directly from the experimental data—one has to estimate them graphically by trial. It is likewise impossible to allocate accurately the proper fraction of the total respiration to the respective systems. The absolute precision with which the calculated line describes the experimental data thus depends to a degree upon the care taken in estimating the constants.

A significant fact emerges from the trials made before arriving at the line shown in Fig. 1. In general it appears that the bend characteristic of the

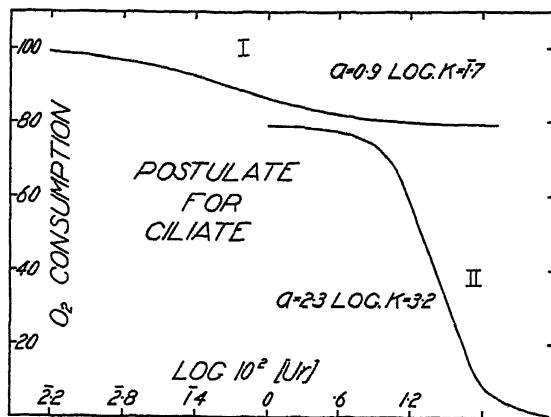


FIG. 3. The theoretical curves expressing as a function of $\log U_r$ the inhibition of each of the two respiratory systems postulated to account for the experimental observations shown in Fig. 1. The total oxygen consumption of the cell in the absence of the inhibitor is taken as 100 units, 20 of these being contributed by system I, the remaining 80 by system III. The constants for the expression of the mass law are given for each curve. At any given urethane concentration the number of units of respiration which have been lost due to the inhibitor is the sum of the losses from each of the two systems. Conversely the respiration still present is the sum of the units still contributed by each system.

data in the figure does not occur in the calculated line *unless* one of the two systems is inhibited appreciably by narcotic concentrations which are too low to affect the other. The respiratory data themselves thus suggest not only that two sites of narcotic action are involved, but also that these two are unequally sensitive to urethane. It immediately follows that if cell division is dependent on the more sensitive system that process would be relatively more sensitive than is the total oxygen consumption. At the concentration marked by the bend the inhibition of the relatively insensitive system is only commencing to be significant (Fig. 3). Since cell division is stopped by this concentration, the inhibition of cell division does appear to parallel inhibition of the relatively sensitive system.

In every respect then the experimental data are satisfied by the conclusion that the total oxygen consumption of *Tetrahymena geleii* is the sum of the consumptions by two separate parallel respiratory systems upon one of which cell division depends. One cannot positively exclude other interpretations, but until these can be tested more rigorously than is possible at present the arrangement outlined above must be considered the most likely one.

Preliminary measurements of the oxygen consumption of both *Colpoda steinii* and *Glaucoma scintillans* suggest that in these forms too the normal respiration actually represents the activity of two respiratory systems.

DISCUSSION

The possibility arises that interference with the rate of cell division may always be the consequence of interference with the operation of some component of the activity system. It is interesting to note in this connection that the consumption of oxygen by the activity system does not of itself ensure cell division. The data in Fig. 1, for example, were obtained with the organisms suspended in a solution of salts in which cell division is not observed. Even in those experiments with nutrient materials present the concentration of organisms used was sufficient to prevent cell division (Ormsbee, 1942), yet evidence for the functioning of the activity system appeared. It is apparently possible to prevent the activity of growth and cell division without interfering with the respiratory system which supports it. At least the interference is not revealed by the experiments reported thus far.

SUMMARY AND CONCLUSIONS

1. The inhibition of oxygen consumption produced by a series of concentrations of ethyl carbamate has been measured in the protozoan *Tetrahymena geleii*.

2. The relation found between the narcotic concentration and its effect on respiration leads to the conclusion that urethane has two distinct modes of action in this cell. The respiratory data can be accurately predicted by assuming that the inhibitor acts on two independent parallel respiratory systems.

3. Complete suppression of cell division in this organism is brought about by approximately 0.1 M urethane.

4. Urethane concentrations up to 0.1 M affect primarily only one of the two postulated respiratory systems. The mechanism of the narcosis of cell division in this organism by urethane thus appears to be inhibition of this "activity" system.

Acknowledgment

The senior author wishes to express his appreciation to Dr. George W. Kidder for his encouragement and aid in the prosecution of this work.

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THE EFFECTS OF URETHANE AND CHLORAL HYDRATE ON OXYGEN CONSUMPTION AND CELL DIVISION IN THE EGG OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

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INTRODUCTION

A quantitative examination of the inhibition of oxygen consumption by urethane in the yeast cell was made recently by Fisher and Stern (1942). These authors concluded that the normal oxygen consumption of yeast was due to the activities of two independent and parallel chains of chemical reactions which differed in their sensitivity toward the narcotic, only one being affected by low concentrations though both were inhibited at higher concentrations. It was shown that inhibition of cell division by urethane in this cell was closely correlated with inhibition of one of the two systems suggested by the respiration experiments. From these data, and others in the literature, the inference was drawn that in yeast, luminous bacteria, and brain cells, narcotics effect a quantitative separation of two respiratory systems. In two of the three cases the separation may be qualitatively distinct as well, for the activities of cell division in one instance, and of light production in the other, appeared to be associated with only one of the two systems. A fractionation of the normal respiration into an "activity" and a "resting" portion, employing these terms in the sense introduced by Stannard (1941), seems to be accomplished by the narcotic. The significance of these findings to narcosis in general has been reviewed briefly by Fisher (1942).

In at least two other cases, frog muscle (Stannard, 1941) and the sea urchin egg (for references see Ballentine, 1940; and Korr, 1937), fractionations of the oxygen consumption similar to that described above for narcotics have been suggested. It was deemed of importance to determine the effects of narcotics in one or more of these cases. The present paper deals with such observations on the egg of the sea urchin. This preparation is of particular interest since a function, cell division, can be initiated at will by fertilization. Two different narcotic agents have been employed, ethyl carbamate (urethane) and chloral hydrate. The effects of these substances on the consumption of oxygen in both the fertilized and unfertilized cells have been determined, as well as the effects on cell division in the fertilized egg.

Materials and Methods

Eggs and sperm of the sea urchin, *Arbacia punctulata*, were obtained essentially as described by Just (1939). The eggs were concentrated by very low speed centrifugation and were then used as a suspension in sea water, the mixture containing 5 to 10 per cent by volume of eggs in the case of fertilized cells or 10 to 20 per cent of unfertilized cells. The percentage of eggs fertilized was uniformly high, averaging 90. The unfertilized eggs used were routinely examined to determine that unintentional fertilization had not taken place.

Oxygen consumption was measured by Warburg's direct method (Dixon, 1934) with air in the gas phase.¹ The vessels were shaken approximately 70 times per minute through an arc of 8 cm. Urethane was added as a solution in sea water directly to the egg suspension at the time the vessels were made up. Chloral hydrate in sea water was tipped into the egg suspension from the onset at the end of the equilibration period. This procedure minimizes the inaccuracy which arises from the fact that chloral hydrate solutions decompose gradually liberating a gas which is not absorbed by the alkali in the Warburg vessels. The gas produced in these experiments became a significant factor only when the chloral hydrate concentration was raised to approximately 0.1 to 0.2 M. Data at higher concentrations than these will not be reported. The respiratory experiments were performed at 25°C. and as a rule were terminated within 3 hours after collecting the eggs.

Since Smith and Clowes (1924 a) have shown that the rate of cell division is relatively independent of pH over the range 6.0–8.3 no attempt was made to control this factor rigidly in the present investigation. Actually it was always within the range of 7.5–8.3, sodium hydroxide being added to the chloral hydrate solutions to neutralize the free acid which was present.

To determine the effect of the inhibitors on cell division, approximately equal numbers of eggs were placed in each of several different concentrations of the narcotics considerably before the first division had occurred. Development was allowed to proceed until, in the controls without inhibitor, the 16 and 32 cell stage had been reached. The time required was approximately 3 hours at the temperature of these experiments, 24°C. Division was then stopped in all of the experimental solutions by the addition of sufficient formalin to make its concentration 0.1 per cent. An index of the average velocity of cell division over the period of exposure to the narcotic was obtained by the method described by Smith and Clowes (1924 b). Each value to be reported is based upon the examination of at least one hundred eggs in each solution. In the present paper the velocity of cell division determined in this way will be expressed as a percentage of the uninhibited control rate. The average difference between duplicate controls is of the order of 1 per cent.

RESULTS

Exclusive of preliminary observations establishing the appropriate range of inhibitor concentration, fertilized sea urchin eggs were subjected to a series of

¹ The rate of diffusion of oxygen from air was adequate as evidenced by the fact that the rate of oxygen consumption in air and in oxygen were found to be identical.

urethane concentrations in five complete experiments. The consequent reductions in the rate of oxygen consumption and of cell division in a typical experiment are shown in Fig. 1. The respiratory data are plotted to test the possibility of describing the inhibitory effects in terms of the law of mass action (*cf.* Fisher and Stern, 1942). The required straight line is not found and consequently the data cannot be described by a simple equation derived from the

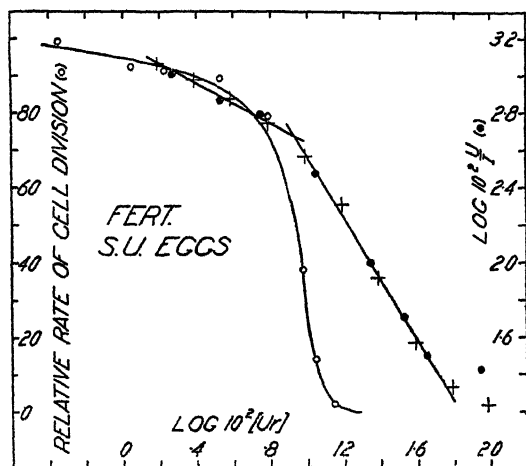


FIG. 1. The effect of different concentrations of ethyl carbamate on the rate of cell division (open circles) and on the rate of oxygen consumption (solid circles) in the fertilized sea urchin egg. U , the "uninhibited" oxygen consumption is the consumption which remains in the presence of a given molar concentration ($[Ur]$) of urethane. I , the "inhibited" oxygen consumption is the difference between U and the normal oxygen consumption. If the respiratory data are capable of description in terms of the law of mass action, it should be found that $\log \frac{U}{I} = \log K - a \log [Ur]$, K and a being constants (*cf.* Fisher and Stern, 1942); *i.e.*, $\log \frac{U}{I}$ plotted as a function of $\log [Ur]$ should give a straight line whose slope is a .

mass law. However, with the exception of the determination at the highest concentration, it is apparent that these data, as plotted, conform closely to two intersecting straight lines. The intersection occurs at a urethane concentration of approximately 0.1 M ($\log 10^2 [Ur] = 1.0$).

Fig. 1 also illustrates the effect of urethane on the rate of cell division. In 0.1 M urethane cell division is practically stopped, although at that concentration the rate of oxygen consumption has only been reduced to about 75 per cent of the normal.

Typical data (single experiment) obtained for *chloral hydrate*, the second

narcotic agent investigated, are given in Fig. 2. Again the respiration observations have been plotted to test the applicability of the mass law. As in the case of urethane they do not at all approximate a single straight line, and consequently cannot be accurately described by a single equation derived from the law of mass action. Instead they too conform reasonably well to two intersecting straight lines. The intersection or bend occurs at a chloral hydrate concentration of approximately 0.0045 M ($\log [\text{C.H.}] 10^4 = 1.65$). This concentration, as shown in the figure, is sufficient to stop cell division although it leaves 45 per cent ($\log \frac{U}{I} 10^2 = 1.91$) of the normal rate of oxygen consumption

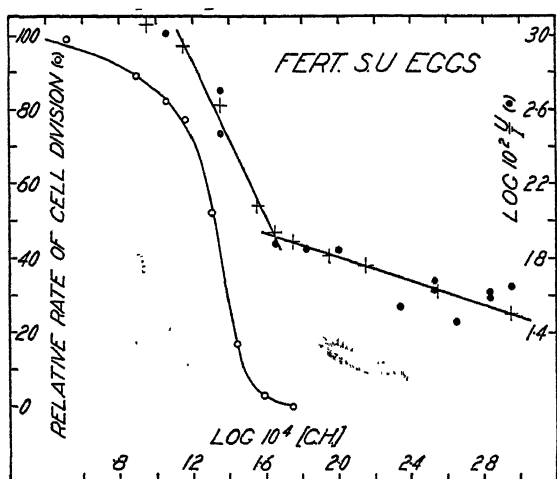


FIG. 2. The effect of chloral hydrate on oxygen consumption and cell division in the fertilized sea urchin egg. Symbols as in Fig. 1.

tion. The experiments with both urethane and chloral hydrate thus exhibit clearly the characteristic ability of narcotics to depress cellular functions unequally. In each case cell division is much more severely affected than is oxygen consumption.

Cognizance must be taken of two factors which might distort an otherwise simple relation between concentration and effect, thus producing as an artifact the form of the respiratory data seen in Figs. 1 and 2. These are (1) irreversible damage, and (2) uncertainty with regard to the equilibrium concentration of the inhibitor due to the uptake of inhibitor by the cells. As judged by the ability to resume normal cleavage upon removal of the inhibitor an exposure longer than 70 minutes to a 0.34 M solution of urethane was required to cause irreversible damage. Complete reversibility of the effects of chloral hydrate was observed after an immersion for 1 hour in a 0.022 M solution and after 30

minutes in a 0.044 M solution. Since the intersections in the plotted respiratory data were found at 0.1 M urethane and at 0.0045 M chloral hydrate respectively they cannot be due in any way to the onset of irreversible changes. The suppression of the oxygen consumption by various concentrations of either inhibitor was constant when the ratio of egg volume to narcotic volume was varied from 1:10 to 1:60. It follows that the quantity of inhibitor bound by the eggs was insignificantly small.

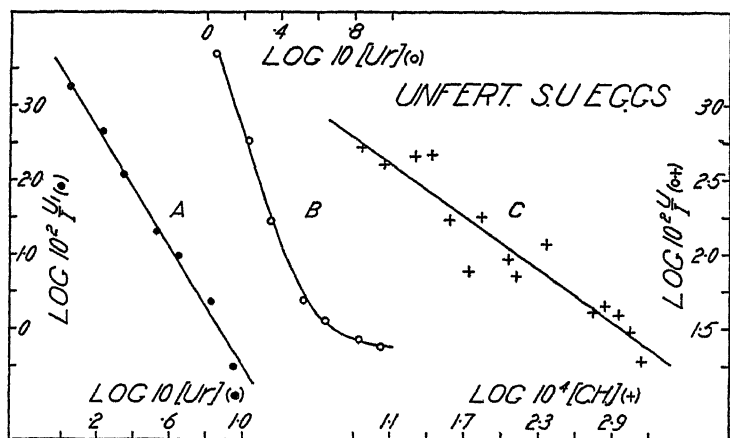


FIG. 3. The effects of urethane and of chloral hydrate on the consumption of oxygen by unfertilized sea urchin eggs. The method of plotting is the same as that described for the respiratory data of Fig. 1. The average observations from six separate experiments on urethane are shown in B; the same data are plotted in A after account is taken of an inhibitor-stable respiration amounting to 19½ per cent of the normal oxygen consumption. Line C typifies the results of the experiments with chloral hydrate. In this case the observations from a single experiment rather than from an average are quoted, in order to illustrate the variability which may occur. See text for details.

Since neither damage nor uncertainty regarding the narcotic concentration is concerned in determining the relation between concentration and effect, it seems that the relations observed must be ascribed to the normal cellular respiratory systems. A striking difference between the actions of the two inhibitors will be noted. Whereas the slope of the line describing the effect of urethane at the higher concentrations is greater than that of the line describing the effect at low concentrations, the exact opposite is true of the lines for chloral hydrate.

The characteristics of the inhibition of oxygen consumption in the unfertilized eggs by the two narcotics are illustrated in Fig. 3. It will be recalled (Fig. 1) that in the fertilized egg 0.1 M urethane reduces the consumption of oxygen to 75 per cent of the normal value and stops cell division. That concentration

in the unfertilized egg has practically no effect (line B, Fig. 3), at most reducing the respiration by only 3 per cent (to $\log \frac{U}{I} 10^2 = 3.5$). The range of urethane concentrations producing graded inhibitions of respiration in the unfertilized egg thus extends upwards from the concentration which suffices to prevent cell division. In contrast the effective concentration range for chloral hydrate is practically identical for the fertilized (Fig. 2) and unfertilized egg (line C, Fig. 3).

Lines B and C of Fig. 3 are straight over most of the range of inhibition which each covers. The slopes of these linear portions are different. They are compared in Table I with the slopes of the intersecting lines found in Figs. 1 and 2.

With each narcotic the value observed for the unfertilized egg is not equal to either of the two observed for the fertilized egg but it is distinctly closer to one of them than to the other. That one in both cases is the value character-

TABLE I
The Apparent Values of a

	Fertilized egg		Unfertilized egg
	Low concentration	High concentration	
Urethane.....	0.53	1.6	3.8
Chloral hydrate.....	2.1	0.32	0.6

istic of the high range of inhibitor concentrations. It applies, as has been noted earlier, to the inhibition which is produced by concentrations greater than those which block cell division.

The points for urethane in Fig. 3B tend to approach asymptotically a level at approximately 19 per cent of the normal rate of oxygen consumption ($\log \frac{U}{I} 10^2 = 1.3$). It is thus suggested that this 19 per cent is urethane-stable.

A straight line is to be expected on the axes used here only when the respiration is completely sensitive to the inhibiting agent. Where a stable fraction exists U (as defined for Figs. 1 and 2) minus this fraction should be employed rather than U alone. The new quantity may be designated U_1 and the results of using it, considering $19\frac{1}{4}$ per cent of the normal oxygen consumption to be unaffected by urethane, are illustrated by line A of Fig. 3. The points conform closely to a straight line, indicating that the mass law provides an adequate description of the effects of urethane on the part of the respiration which is sensitive to this inhibitor. Line B was calculated using the constants obtained from line A and taking into account the narcotic-stable fraction.

The experiments with chloral hydrate do not reveal any narcotic-stable oxygen consumption. This is undoubtedly due to the fact that the gas production referred to earlier prevented the use of the high concentrations which would be necessary to demonstrate it.

Cell Division

For comparison with the data already presented the observations on cell division which were included in Figs. 1 and 2 respectively, appear again in Fig. 4 where they have been plotted to test the applicability of the law of mass action. It is apparent that in neither case is the required straight line found. Further

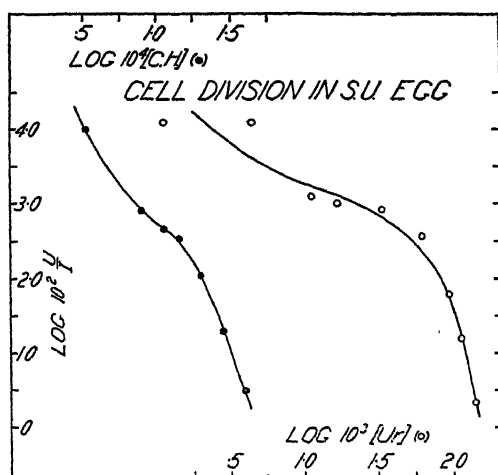


FIG. 4. The possibility is tested here of describing the data for cell division from Figs. 1 and 2 by an expression of the law of mass action. See text for further details.

experiments are necessary in this connection since it is possible that the shape of the curves may be affected by the criterion of rate of cell division which was used.

The general conclusions to be drawn from the foregoing observations must take into account the following principal facts: (1) In unfertilized eggs which do not divide the mass law plot of the respiratory data shows only one line, while (2) in the fertilized egg which does divide two intersecting lines appear. (3) Complete inhibition of one function, cell division, occurs at narcotic concentrations which cause a relatively smaller effect on another function, oxygen consumption. (4) Where two intersecting lines are found in the mass law plot, the slope of the line at the lower inhibitor concentrations is greater than that of the line at the higher concentrations for one narcotic, but is just the opposite for the other. (5) For both narcotics the slope of the line for un-

fertilized eggs tends to approach in value the slope of the line covering the higher concentration range of the same inhibitor for the fertilized eggs.

It will be noted at once from point (1) above, that the effects of narcotics in the unfertilized egg can be completely accounted for if there exists only a single site of narcotic action in those cells. However, on the basis of a single site it seems quite impossible to account in a reasonable manner for the observations on the fertilized egg (points (2) to (5)). If two such sites were involved, the next simplest possibility, they might be in series in a single chain of respiratory reactions, or each might be in one of two parallel chains which together made up the total oxygen consumption of the cell. With two sites of action in either series or in parallel, and with the effect of the inhibitor at each governed by the mass law, calculation shows that it is still possible for the total respiration of the cell to give practically a straight line when plotted as in Figs. 1 and 2. As far as the immediate considerations are concerned therefore, the narcotics could conceivably act at two sites in the unfertilized as well as in the fertilized egg. The approximation to a single straight line happens, however, only when the two sites are affected over essentially the same range of concentration. If they are affected by significantly different ranges of concentration, a more likely situation, the resultant of the two effects differs very markedly from a single straight line, just as the data for the fertilized egg do in Figs. 1 and 2.

Suppose then that there are two sites in series and that they are acted upon by different ranges of inhibitor concentration. As the concentration gradually rises, the degree of inhibition will change with the concentration at a rate determined primarily by the characteristics of the more sensitive of the two sites. Let the concentration now become great enough to cause an effect at the second one. The simplest conditions which can be imagined require that the metabolism already partially inhibited at one stage shall be still further reduced at the second. It thus follows that when the concentration has been raised sufficiently to cause inhibition at both sites, the degree of inhibition must increase more rapidly with concentration thereafter, than it would if the narcotic were acting at only a single site. This is the picture given by urethane in Fig. 1.

The degree of inhibition of oxygen consumption as measured by $\log \frac{U}{I}$ increases more rapidly at concentrations above the point of intersection, than it does at concentrations below that point. The degree of inhibition with chloral hydrate on the other hand changes less rapidly with concentrations above the point of intersection than it does at concentrations below that point. While a final statement is perhaps impossible it is difficult to see how this could happen if there were two sites and these were in series. It is likewise difficult if not actually impossible at present to visualize a mechanism which would account for points (3) and (5) on the basis of two sites of action in series.

These experiments indicate in general then that there are at least two distinct

sites for the action of chloral hydrate and of urethane, but that these are not likely to be in series.

On the other hand, the properties of a parallel arrangement of the two sites are such as indicate that this arrangement would account for all of the observations recorded here. It requires that the total oxygen consumption of the cell be the sum of the oxygen consumptions by two separate parallel chains or systems of respiratory reactions. Each includes a step which is inhibited by narcotics and since these two sites are distinct there will be a separate expression of the mass law describing the action of the narcotics at each site. The form of the graph to be expected when the total oxygen consumption of such a preparation is plotted as in Figs. 1 and 2, can readily be calculated. As was stated above it may approximate a single straight line (such as was found for the unfertilized egg) or it may instead approximate two intersecting straight lines as do the data for the fertilized eggs. The parallel arrangement permits the possibility of the observations referred to under point (4) above although the series arrangement does not, as has been noted.

Two straight lines similar to those in Figs. 1 and 2 result when the affinities of the two sites for inhibitors are sufficiently different, that is, when one of the chains is appreciably inhibited by concentrations too low to affect the other. In this case the per cent inhibition of the more sensitive system increases more rapidly as the concentration is raised than does the per cent inhibition of the total oxygen consumption (= sum of oxygen consumption by the two separate chains). The data for the fertilized egg thus suggest not only that two respiratory systems in parallel are responsible for the observed oxygen consumption, but also that one of the two must be affected at significantly lower inhibitor concentrations than is the other. Cell division is inhibited more rapidly as the concentration of narcotic rises, than is the total oxygen consumption of the egg. Inhibition of cell division then, parallels inhibition of the more sensitive of the respiratory systems more closely than it does inhibition of the total oxygen consumption. It may be that the oxidative metabolism required for cell division is mediated by this chain rather than the one affected only at higher concentrations of narcotic. Because of the correlation if not actual connection between the activity of cell division and one of the chains of respiratory reactions that one, the more sensitive of the two, will henceforth be referred to as the "activity" system or chain. The term "basal" or "resting" will be applied to the remaining system.

Point (5) dealing with the slopes of the lines on the double log axes, leads to a conclusion regarding the identity of the narcotic-sensitive respiratory system active in the unfertilized egg and the basal system of the fertilized cell. When only a single site of narcotic action exists, the slope of the line on the double log axes gives directly the value of a in the expression of the mass law which describes the data. If there are two sites and two lines result when the total

oxygen consumption is used in making the double log plot, then the slope of each line may differ very appreciably (50 to 60 per cent) from the value of a which primarily determines it. The values of these slopes therefore will be referred to as "apparent" values of a to distinguish them from the true values. The slopes for the fertilized egg which are listed in Table I are apparent values while those for the unfertilized egg, since they were determined from single lines, may be true values. In general the apparent values are always less than the true values. The latter for the fertilized egg in the high concentration range must then be much closer to the values for the unfertilized egg than are the apparent values listed in the table.

If the mass law constants are such as cause the data to approximate a single line even when two parallel sites are involved, then the slope of this line, the apparent a , is nearly the average of the two separate values of a which are concerned. It is evident from Table I that the slopes for the unfertilized egg are not the average of the respective two slopes for the fertilized egg. If two sites of narcotic action exist in the unfertilized egg these cannot be the two which operate in the fertilized egg. Of the several possibilities which exist it is simplest to consider that there is only one site in the unfertilized cell so that the slope in this case gives the true a . It has already been noted that the slope in the unfertilized cell is much more similar to the true a for the basal system than it is to that of the activity system. There is a real possibility therefore that the narcotic-sensitive respiratory system of the unfertilized cell may be identical with the basal system of the fertilized one. An evaluation of the possibility requires a determination of the true a 's for the fertilized cells.

Unfortunately it is not possible to calculate from the experimental data the constants for the two expressions of the mass law required to describe the effects of each inhibitor on the two respiratory systems of the fertilized egg. They can be estimated graphically, however, by trial. Values determined in this way for the fertilized egg, along with those calculated for the unfertilized egg from the data in Fig. 3 are given in Table II. The correspondence between the true value of a for the basal system and that for the unfertilized egg is striking, suggesting strongly that the former system and the one operating in the unfertilized eggs are in fact identical.

The graphic method produces also the estimate that 40 per cent of the total oxygen consumption of the fertilized egg is mediated by the activity system, the remaining 60 per cent by the basal system.

The curves corresponding to the constants given in Table II are shown in Fig. 5. A theoretical set of data may be constructed from them (Fisher and Stern, 1942) for comparison with the experimental data. The large crosses which appear in Figs. 1 and 2 are points calculated in this way. It is evident that they describe the data in an adequate manner. The conclusion that there are two parallel respiratory systems in the fertilized egg is thus quantitatively

as well as qualitatively justifiable. In the unfertilized egg the indication is that only a single respiratory chain is present and that it is identical with the

TABLE II

A Comparison of the Values of the Mass Law Constants for the Inhibition of Oxygen Consumption in Fertilized and Unfertilized Eggs of the Sea Urchin

	"Activity" system		"Basal" system	
	Fertilized	Unfertilized	Fertilized	Unfertilized
Urethane:				
a	0.5	?	3.0	4.0
$\log K$	$\bar{1}.7$?	$\bar{2}.0$	$\bar{3}.48$
Chloral hydrate:				
a	6.0	?	0.5	0.6
$\log K$	$\bar{16}.9$?	$\bar{1}.25$	$\bar{2}.87$

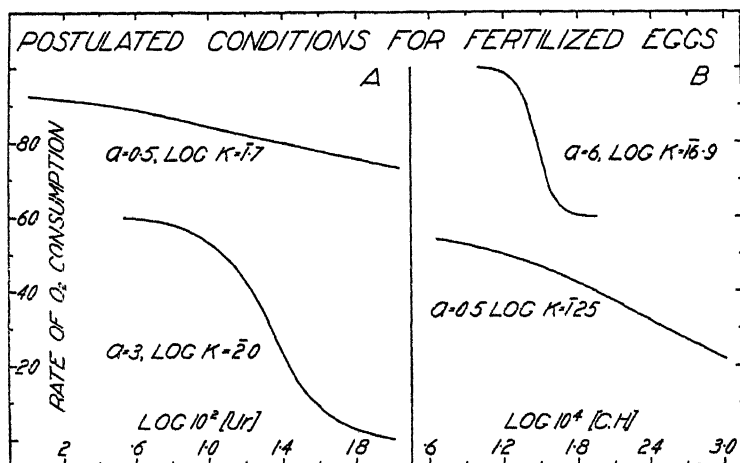


FIG. 5. A. The inhibition of the two postulated systems as a function of $\log [\text{Ur}]$. B. The inhibition of the two postulated systems as a function of $\log [\text{C.H.}]$. The upper curves in both cases refer to the inhibition of a respiratory system which will be designated as the activity system. Similarly the lower curves indicate the effect on the basal system. The normal rate of oxygen consumption is assumed to be 100 units. Forty of these are contributed by the activity system, the remainder by the basal system.

basal system. The activity system is apparently not functional until the cells have been fertilized, an additional circumstance which points to a specific relation between it and the process of cell division which is also initiated by fertilization.

In connection with the conclusions reached above, the recent observations by Krahle, Keltch, Neubeck and Clowes (1941) on the sea urchin egg are of great interest. These investigators have reported that azide will remove only 50 per cent of the total oxygen consumption of the fertilized egg. Moreover the concentration of this inhibitor required to stop cell division is just sufficient to produce the maximum arrest of oxygen consumption. The similarity between the conclusions reached above and those to be drawn from these experiments with azide are obvious and need no comment.

It will perhaps have been noted that the amount of oxygen consumed by the basal system has not been compared in the foregoing with that consumed by the unfertilized eggs. Ultimately the analysis of the metabolic changes upon fertilization must include this comparison. The result of it can hardly alter the general conclusions now reached, however, and consequently it will not be discussed in the present communication.

SUMMARY

The effects of a series of concentrations of the narcotics, ethyl carbamate and chloral hydrate, have been determined on the consumption of oxygen by fertilized and unfertilized eggs of the sea urchin *Arbacia punctulata*. In the fertilized eggs the effects of the two inhibitors on cell division were also examined. The following observations were made:

1. Assuming that the narcotic acts upon a single catalyst in the unfertilized egg the degree to which the consumption of oxygen is inhibited in this resting cell can be related to the narcotic concentration by an expression derived from the law of mass action.

2. To account for the relation between the concentration of the narcotic and its effect on respiration in the fertilized eggs, it is necessary to conclude that in them the narcotic acts on two parallel respiratory systems. The experimental data can be quantitatively predicted (1) if the reaction of the narcotic on the two systems is governed by the law of mass action and (2) if 40 per cent of the oxygen consumption is mediated by one system, the "activity" system, and the remainder by the other, the "resting" or "basal" system.

3. The mass law constants applying to the resting system in the fertilized egg are similar to those for the single system functioning in the unfertilized egg so that these two respiratory systems are probably identical.

4. The concentrations of the narcotics just sufficient to abolish cell division affect primarily the activity system, the existence of which was inferred from the respiratory experiments. It is concluded that normal cell division requires specifically the normal function of the activity system, that in fact the energy for cell division is made available through that system.

We wish to record our appreciation of the assistance given by Mr. G. R.

Norman in the early experiments, and our thanks to the Banting Foundation, Toronto, for continued support.

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CORRECTIONS

In Vol. 27, No. 5, May 20, 1944, page 403, in the eighth line from the bottom of the page, the comma after "intensity" should be a semicolon.

On page 413, in the second formula from the bottom of the page, for " $\sigma_r = \frac{\bar{V}}{\sqrt{2r}}$ " read " $\sigma_r = \frac{\bar{V}}{\sqrt{2n}}$."

On the same page, formula 2 should read " $\sigma_{1\sigma_1} = \frac{\bar{\sigma}_1}{\sqrt{2}} \sqrt{1-r}$."

On page 414, line 3, at the end of the line add "or" to read "of the level of I or of F ."

On page 422, in the first line below the figure legend, for "illuminate" read "illuminated."

On page 430, line 22, for "lighteb dars" read "lighted bars."

THE KINETICS OF IN VIVO HEMOLYTIC SYSTEMS

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The purpose of this paper is to explore the possibilities of treating the contents of the blood stream as a hemolytic system in which a more or less steady state is maintained by the production of new red cells to replace those which are destroyed. The attempt will raise both new questions and questions which have been the subject of investigation already, and I realize that it will often do no more than outline the problems still to be solved. In their essentials, these problems are those of the nature of the *in vivo* lysins, the nature of their accelerators and inhibitors, the rules which apply to the mixtures of lysins, accelerators, and inhibitors which compose the *in vivo* hemolytic systems, and the kinetics which describe the way in which red cell destruction by the hemolytic mixtures is balanced by the production of new red cells.

I. The Lytic Effect of Mixtures of Lysins, Accelerators, and Inhibitors

In vivo hemolytic systems consist of mixtures of many lysins, accelerators, and inhibitors, and relations must exist between the hemolytic activity of these mixtures and the activity of the lysins acting singly, accelerated or inhibited by individual accelerators and inhibitors. One would expect the effect of the individual components to be approximately additive, although reactions between them might enhance or depress their activities. This is what seems to occur in mixtures of lysins (Ponder, 1933).

Two types of experiment are suitable for studying the combined effects of lysins, accelerators, and inhibitors. In the first the effects are obtained in systems in which lysis occurs in short times, while the second applies to systems more closely resembling *in vivo* systems, in which the lysin exists in small and nearly asymptotic concentration.

1. A standard time-dilution curve is obtained at 37°C. for systems containing 0.8 ml. of lysin, 0.8 ml. of 1 per cent NaCl (saline), and 0.4 ml. of red cell suspension (the thrice washed cells of 1 ml. of blood suspended in 20 ml. of saline). Systems in which the accelerators or inhibitors are present in various amounts are then prepared *e.g.*, a system might contain 0.8 ml. of lysin, 0.4 ml. of an accelerator, 0.4 ml. of saline, and 0.4 ml. of cell suspension. If c_s is the concentration of lysin in the standard system which takes the same time for complete hemolysis as a concentration c_a in the system containing the accelerator, or c_i in the system containing the inhibitor, $c_a/c_s = R_a$ for the accelerator, and $c_i/c_s = R_i$ for the inhibitor.

Systems containing both the accelerator and the inhibitor can be prepared by taking 0.8 ml. of lysin, 0.4 ml. of the accelerator, 0.4 ml. of the inhibitor, and adding 0.4 ml. of the cell suspension. The effect of the accelerator and the inhibitor acting together is expressed, by reference to the standard time-dilution curve, as R_{ai} . Innumerable ways will suggest themselves for combining accelerators and inhibitors in systems which are comparable to standard systems. The general problem is whether R_{ai} is the same as the calculated net effect of the individual effects R_a and R_i , which, because of the way in which R is measured, is equal to $(R_a)(R_i)$.

Table I shows the result of a typical experiment at 37°C. in which the lysin is saponin, the accelerator 1 mm/l. indol, and the inhibitor plasma diluted 1 in 100. In this system the correspondence between R_{ai} and $(R_a)(R_i)$ is quite good, although the component of acceleration seems to be favored, especially

TABLE I

Saponin 1 in	Accelerator and inhibitor	R_a	R_i	R_{ai}	$R_{calc.}$
10,000	Indol, 1 mm/l., 0.4 ml. Plasma 1/100, 0.4 ml.	0.70	1.70	1.92	1.19
15,000		0.73	1.67	1.06	1.22
20,000		0.70	1.60	1.00	1.12
25,000	Indol, 1 mm/l., 0.2 ml. Plasma 1/1000, 0.2 ml.	0.76	1.27	0.91	0.96
30,000		0.78	1.27	0.95	0.99
35,000	Indol, 1 mm/l., 0.2 ml. Plasma 1/100, 0.05 ml.	0.74	1.14	0.84	0.85

in the systems in which lysis is rapid. This may be due to some such effect as the accelerator being more rapidly concentrated at the cell surfaces than the inhibitor. Speaking generally, the results obtained by this method are the same as those obtained by the method next to be described, which measures the values of R_a , R_i , and R_{ai} at the asymptotes of the respective time-dilution curves.

2. The asymptote of the standard time-dilution curve is found in the usual way for systems containing 0.8 ml. of lysin, 0.8 ml. of saline, and 0.4 ml. of red cell suspension. In a similar way, the asymptotes are found for (a) a system containing 0.8 ml. of lysin in various dilutions, 0.4 ml. of an accelerator, 0.4 ml. of saline, and 0.4 ml. of suspension, and (b) a system containing 0.8 ml. of lysin, 0.4 ml. of inhibitor, 0.4 ml. of saline, and 0.4 ml. of suspension. For practical purposes, the concentration of lysin which produces complete lysis in 300 minutes may be taken as the asymptotic concentration. The position of these two asymptotes and that for the standard curve determines R_a and R_i . A series of systems containing 0.8 ml. of lysin, 0.4 ml. of accelerator, 0.4 ml. of inhibitor, and 0.4 ml. of cell suspension is then set up, and the asymptote for

the time-dilution curve is found. This gives R_{ai} . Table II shows the results for three experiments of this kind.

When we take these results together with others (accelerators, Ponder, 1941; inhibitors, Ponder, 1943; and mixed lysins, Ponder, 1933) I think that it can be said that the behavior of mixtures of accelerators, inhibitors, and lysins is fairly well established, provided that the systems are relatively simple. The net effects can usually be obtained by appropriate calculations in terms of R -values, although sometimes enhancement or partial neutralization of effects occurs. When dealing with hemolysins *in vivo*, however, we are dealing with a mixture of unknown lysins, and in the case of any one inhibitory or acceleratory substance the question must always arise as to whether its effect on an *in vitro* system containing lysins such as saponin, a bile salt, or a soap, is any guide to its effect on the *in vivo* system of unknown composition. At present there is no way of answering this question except by direct experi-

TABLE II

Lysin	Accelerator and inhibitor	R_a	R_i	R_{ai}	$R_{calc.}$
Saponin	Indol, 1 mM/l., 0.4 ml. Plasma 1/100, 0.4 ml.	0.75	3.0	2.14	2.25
Taurocholate	Same	0.33	1.5	0.50	0.50
Oleate	Same	0.30	2.5	0.75	0.70

ment, which must take the form of seeing whether the substance increases or decreases red cell destruction in the intact animal. In the few cases in which the experiments have been done (*e.g.*, indol, by Rhoads and his collaborators, 1938; phenothiazine, by Collier and Allen, 1942; quinine, by Ponder and Abels, 1936) the substances which were found to be accelerators *in vitro* were found to be accelerators *in vivo* also.

II. The Nature of the Intravascular Lysins

Principally because of Rous and Robertson's observation (1917) that fragmentation plays such an important part in red cell destruction *in vivo*, evidence for the existence of intravascular lysins in normal blood has been less energetically sought than it might have been. Rous says "At the present time (1923) the thesis that hemolysis is concerned in normal blood destruction must be looked upon as unproven. . . . Only two methods have so far been discovered whereby worn out cells leave the circulation, namely, phagocytosis and fragmentation. . . . Both processes may quite possibly be of little importance as compared with some other unrecognized as yet. One is privileged to believe as one wishes about the matter, but scarcely to draw conclusions." Even today, it must be admitted that the evidence for the destruction of red cells

by lytic processes is not very impressive as long as we confine ourselves to normal material; if evidence obtained from pathological material is admitted, on the other hand, there never has been much doubt about the matter (see Rous's review). The weight of the evidence will appear greater, moreover, if we bear in mind that hemolytic processes may exist *in vivo* although the normal method of red cell destruction is fragmentation and phagocytosis. Suppose that the effective concentration of intravascular lysis is c_1 and that in this concentration lysis would take 50 days. If there is a simultaneous process of fragmentation by means of which red cell destruction is effected in 30 days, fragmentation will appear as the method of normal destruction, although the slower process of lysis is operating all the time. If the concentration c_1 were to double, however, the new concentration c_2 might be able to produce lysis in 25 days, and the destruction of the cells by the hemolytic process would occur before the destruction by fragmentation. Fragmentation and phagocytosis as the usual methods of red cell destruction are thus by no means incompatible with the continual operation of *in vivo* lysins, the effects of which may become prominent if their concentration increases.

The three normal *in vivo* hemolytic processes for which there is substantial evidence involve (1) the action of the soaps in chyle after a meal rich in fat, (2) the action of the spleen in modifying red cells so as to aid in their destruction, and (3) the hemolytic effect of substances derived from tissues such as lung, liver, and spleen.

1. Freeman and Johnson (1940), and Loewy, Freeman, Marchello, and Johnson (1943), have shown that the soap content of the chyle rises to 3 to 6 mg. per ml. during rapid fat absorption, and that this quantity of soap is sufficient to bring about a certain amount of hemolysis when it enters the blood stream. During fasting, soaps are found in amounts too small to cause experimentally demonstrable hemolysis, but when the individual is eating an ordinary diet their continual action probably provides a normal mechanism for red cell destruction. Again it should be emphasized that the important thing from the standpoint of this discussion is the *existence* of the *in vivo* hemolytic system, even although the concentration of lysin in it is not sufficient to hemolyze a single cell before the cell's life is terminated by other processes (fragmentation, phagocytosis, etc.). If the hemolytic system exists normally, there are always the possibilities (a) that the concentration of lysin will increase and that intravascular lysis will become an important mode of cell destruction, and (b) that in the presence of an accelerator, or because of the reduction in the concentration of an inhibitor, the lysin will become more effective than it was previously. Whenever one can show that an increase in the concentration of a normally occurring blood constituent results in intravascular hemolysis, I think that one can assume that a normal *in vivo* hemolytic system exists; Johnson and his collaborators have done this for the soaps, and by the same sort of

reasoning there is satisfactory evidence for the existence of a hemolytic system containing the bile salts.

2. The evidence for the spleen being an organ in which red cells are destroyed or prepared for destruction has been reviewed by Rous (1923), by Krumbhaar (1926), and more recently by Dameshek (1941, 1942). The frequent observation that red cell resistance to certain forms of lysis is increased *in vitro* after splenectomy was shown by Gordon and Kleinberg (1937) to correspond to an increased resistance to destruction *in vivo* in the guinea pig, and the increased resistance to hypotonic saline is due to the cell being able to attain a greater volume before it hemolyzes (Gordon, Kleinberg, and Ponder, 1937). The removal of the spleen is accordingly followed by an alteration in the structure of the red cell, and the increase in resistance to lysis by taurocholate (although not to lysis by saponin) points to the same conclusion. After splenectomy in man the alteration in structure is accompanied by a change in shape, the red cell population containing an increased number of flat cells ("platycytes," some of which may even be "target cells," Singer, Miller, and Dameshek, 1941). The reason for these shape changes is still obscure, but according to the view which prevails at the moment, the spleen produces substances which tend to make red cells less flat and more spheroidal, the continued action of which may eventually produce spheres and hemolysis. Removal of the spleen with its spherocyte-producing substances thus results in a less spheroidal, or flatter, population.¹ The possible nature of these substances has been dis-

¹ An extreme statement of this point of view would be that the red cell is originally produced as a very flat body ("platycyte") with an a/b ratio of, say, 6 to 1, that as a result of the continued action of substances produced by the spleen and perhaps by other tissues it "tends to spherocytosis" by becoming less and less eccentric as it grows older, and it finally becomes a spherocyte and hemolyzes. The whole process would have to take weeks, starting in the marrow where the youngest red cells, although not yet in circulation, would nevertheless be exposed to the action of circulating lysins, and the mean diameter and thickness of the cell as ordinarily measured would correspond to the diameter and thickness of the cell of mean age, thinner cells being younger and thicker cells older. So far as the kinetics of intravascular hemolysis are concerned, there is no objection to this hypothesis, which would correspond very well to the transformation of S to S' described in section IV, provided that the cell shape were a function of S or S' . The objection to it is that lysins do not usually convert red cells into spheres with a series of biconcave forms of varying eccentricity as intermediates. At least, when studied *in vitro*, the intermediates are crenated or thorn-apple forms (Ponder, 1935, 1942), and these are not often seen in the circulation. It is possible that a lysin might produce regular forms of diminishing eccentricity instead of crenated forms if it were to act slowly enough, but until such a phenomenon is shown to exist the chain of evidence is incomplete. (Some evidence for it may be found in a paper by Tigertt, Duncan, and Hight (1940), but the red cell diameters were measured in dried films.) One might put forward a hypothesis to the effect that the red cell is

cussed by Singer (1940, 1941), Singer, Miller, and Dameshek (1941), Ham and Castle (1940), and Dameshek and Schwartz (1938). Bergenhem and Fahraeus (1936) have shown that serum contains an enzyme which splits off a substance from the serum lipoids after some hours *in vitro*; this substance can be adsorbed on the red cell, and produces a change from the biconcave to the spherical form. The enzymatic process proceeds most rapidly at 42°C. and at pH = 7.2, and is inactivated at 56°C. Bergenhem and Fahraeus have identified the substance as lysolecithin, small amounts of which produce spherocytosis and larger amounts of which produce hemolysis (*cf.* the action of lecithin, Ponder, 1935, 1942). It is possible that the spherocyte-producing substance of the spleen is actually lysolecithin.²

3. Maegraith, Findlay, and Martin (1943 *a, b*) have recently described a lytic agent, which they think is an erythrocytase, in a variety of human and animal tissues. Its presence can be demonstrated by adding small pieces of washed tissue to a washed red cell suspension and incubating for 24 hours at

initially a very flat body, that the continued action of lysins from the spleen and elsewhere makes it less and less eccentric, but that fragmentation normally occurs before the stage of crenation and sphere formation is reached. This would account for the absence of crenated and spherical cells from normal blood, and only when there was an increase in the activity of *in vivo* hemolytic activity might we expect to see circulating spherical forms and the forms (spheroidal or crenated) preceding them. This would be an interesting working hypothesis, but I think that it should be borne in mind that the spleen may also control the shape of the red cells as produced at their source.

A different mechanism by means of which decreased eccentricity may be produced is that described by Ham and Castle (1940). Under conditions of stasis, such as probably occur in the spleen, red cells swell as a result of changes in permeability which follow on the accumulation of metabolic products, and in swelling they tend to become first less eccentric and later spherical. This change in form is well recognized (Castle and Daland, 1937; Ponder, 1937), and is due to quite a different mechanism from that responsible for the disk-sphere transformation produced by lysins like lysolecithin. While it might be effective in producing red cell destruction, especially if the *a/b* ratio for the cell is less than normal, it is not necessary to think of it as a mechanism which regulates the mean shape of the red cell in the circulating blood.

² Great caution has to be exercised in identifying substances extracted from blood and tissue, and the fact that a lytic substance can be extracted cannot be taken as evidence that it exists, as a lysin, in the blood or tissue *in vivo*. A great number of fatty acids, soaps, and related substances can be obtained by various extraction processes, but before extraction these are probably combined with other tissue components in such a way as to render them non-lytic. The apparent amount and nature of these extractable substances depends on such details as the temperature and pH at which the extraction is carried out, and different mixtures of substances, some lytic, some inhibitory, can be obtained by using different concentrations of alcohol; *e.g.*, 50 per cent, 75 per cent, and so on.

37°C. The lysin is described as being species specific, destroyed by heating to 80°C. for 5 minutes, and inhibited by plasma in dilutions as great as 1 in 1000. It seems to bear a relation to the tissue lysins originally described by Metchnikoff, of which an excellent account was given by Weil in 1907. Most of Weil's work was done with kidney tissue, freed from blood by perfusion, but he also used a variety of tumor tissue, some of which was necrotic. He found that normal tissue, if chopped up in 10 times its weight of saline and stirred for some hours, yields substances in saline extract which are hemolytic for the washed red cells of the same animal (2 hours at 37°C., followed by 18 hours in the ice box; Weil did not ascertain whether the lysin hemolyzed the cells of other species as well as the cells of the same species). Liver and kidney extracts were found to contain a variable amount of lysin, which is activated by the addition of red cell stromata, inhibited by serum, and which can be shown to become bound to the surfaces of the red cells of the system. Weil points out, however, that the situation is more complex than appears at first sight, for some of the lytic substances which appear in extracts of incompletely washed tissues are derived from the residual blood. Extracts of non-necrotic tumors act like extracts of normal tissues, but necrotic tissue contains entirely different lytic substances in addition. These are diffusible, and are presumably products of necrosis; Weil suggests that the frequent anemia which accompanies malignancy in its later stages may be due in part to the hemolytic action of these products of necrosis.

I have had no difficulty in confirming Maegraith, Findlay, and Martin's results in so far as the lytic effect of pieces of tissue is concerned. Most of my material has been either rat lung or human tissue removed in the operating room. All procedures, including the removal of the tissue, must be carried out aseptically.³ The tissue is cut into pieces about 4 mm. by 2 mm., and these are washed several times in saline. Several of the pieces are transferred to each of a series of test tubes, to which 2 ml. of a suspension of washed red cells are added; the cells may be those of the same species or of another species, and buffers, inhibitory agents, etc., can also be added as the experiment requires. The tubes are kept in a water bath at 37°C. with occasional shaking, and the results are read after 18 to 24 hours. Lysis rarely begins before 12 hours have elapsed, but proceeds fairly rapidly once it starts. The existence

³ These experiments must be carried out with the strictest aseptic precautions, and the hemolytic systems must be shown to give no growth on blood agar plates at the time the results are read. I have found this degree of sterility very difficult to attain, for animal tissues often contain organisms which grow under the almost ideal conditions of incubation for 24 hours at 37°C. in the presence of autolyzing tissue, and hemolytic contaminants can be derived even from the air, at least in Mineola. One large gram-positive "hay bacillus" is strongly lytic on culture under the conditions of these experiments.

of such a long "latent period" in itself suggests that the lytic material is derived from precursors in the tissue.

The lysin (or lysins) does not diffuse through either collodion or cellophane, and is destroyed by heating to 60°C. for 5 minutes. It has a pH optimum at about 7.1; at pH = 6.5 and 8.1 (phosphate buffers) some lytic activity can be observed, but at pH 4.5, 5.8, and 8.5 the lysin is apparently inactive. These observations are in agreement with those of Maegraith, Findlay, and Martin. The point upon which I cannot agree with them is that of the species specificity. I have obtained lysis of human cells with pieces of rat lung and lysis of rat cells with pieces of human thyroid, and, in general, have not found that the lytic material affects the cells of the same species only.⁴

The absence of species specificity puts the matter in a different light, for if we are dealing with a non-specific lysin we have to consider the possibility that it is lysolecithin or a substance related to it. If pieces of rat lung, etc., which prove to be lytic after 12 to 24 hours are incubated at 37°C. in saline for 12 hours, quite large amounts of lysolecithin (or, more properly, the substance identified as lysolecithin) can be extracted from them by the process described by Singer (1940) for the extraction of lysolecithin from serum. The amount is about the same as that contained in normal human plasma, and it is sufficient to produce lysis of a suspension of washed red cells within the second 12 hours of the 24 hour experiment.⁵ Since lysolecithin is produced by an enzymatic process with a pH optimum of about 7.2, both the heat sensitivity of the tissue lysin, the effect of pH on it, and, indeed, all of its properties which I have observed, would be explainable on the assumption that it is lysolecithin or a related substance. If so, lysolecithin is probably a normal intravascular lysin produced by the tissues in a wide-spread manner, and not in the spleen only. Its lytic activity would be dependent on its concentration in the plasma, together with the concentration and effectiveness of its plasma inhibitors.⁶

⁴ An apparent specificity may be due to the cells of one species being less resistant to a lysin than the cells of another; *e.g.*, lysolecithin, in sufficient dilution, would appear to be specifically hemolytic for dog cells if its effects were observed on suspensions of the red cells of man and of the dog. It is possible, of course, that both species-specific and non-species-specific lysins are present at the same time, and that sometimes the former are so active that the effects of the latter pass unnoticed by comparison. I have never observed this state of affairs. The question as to the species specificity of the tissue lysins played quite an important part in the early history of the subject (see Weil, 1907), and it was ultimately decided that the lysins then under discussion, at any rate, were non-specific.

⁵ The hemoglobin of the hemolytic system may be converted to a brownish derivative both by the tissue lysins and by the extracted substances.

⁶ A real difficulty, however, lies in the smallness of the quantity of lysolecithin which can be extracted from plasma by Singer's method. If one dissolves the lytic

III. The Nature of the Plasma Inhibitors

The nature of the inhibitory substances contained in plasma and serum has been under investigation ever since the inhibitory effects were first described. Both the plasma proteins and the plasma lipoids are inhibitory, and the first problem is to find how much of the inhibition is due to the former and how much to the latter. This is a point on which observers have held different views, some attributing most of the inhibition to the proteins, and others believing that the lipoids are principally responsible. The second question is whether there are diffusible inhibitors in addition, for a powerful inhibitor of digitonin hemolysis, the quantity of which is stated to be dependent on the intake of thiamine, has been recently described (Farley, 1939, 1942; Horwitz and Farley, 1940).

1. *Inhibition Produced by Plasma Proteins and Lipoids.*—There are two ways in which we may identify the inhibitory substances and determine their inhibitory powers. The first is to fractionate the plasma and to measure the inhibition produced by the various fractions; the second is to start with pure substances, such as serum albumin, fibrinogen, cholesterol, etc., in known concentration, and to compare the inhibitory effect of each with that of plasma as a whole.

All the inhibitory substances of plasma seem to be non-diffusible, and I have been unable to obtain evidence for the existence of the diffusible inhibitors described by Farley (see below).

A partial separation of the constituents of plasma can be effected by adding dilute acetic acid to plasma diluted 1 in 10 with saline, until the pH is about

material extracted from 2 ml. of normal human plasma, after incubation at 37°C. for 24 hours, in 2 ml. of saline, one obtains a solution 0.2 ml. of which usually completely hemolyzes 0.1 ml. of standard red cell suspension (the thrice washed cells of 1 ml. of human blood finally suspended in 20 ml. of saline) in 24 hours *in vitro* at 37°C. Now Ponder, Hyman, and White (1941) and Ponder and Hyman (1943) have shown that the activity of a lysin in a system containing whole blood is very much less than its activity in an *in vitro* system such as this, even when allowances are made for the maintenance of the lysin at a constant level, as it is maintained *in vivo*. This relatively small activity in the system containing whole blood is due to the greater concentration of red cells, to some of the lysin being used up in reacting with the vessel walls, and to the action of the plasma inhibitors. The inhibitory power of normal human plasma for lysolecithin is such that plasma diluted 1 in 100 gives an *R*-value of between 1.5 and 2.0, and so is of the same order as the inhibitory power for saponin or digitonin hemolysis. The extracted lysolecithin of plasma, a feeble lysin even *in vitro*, would therefore be so much feebler *in vivo* as to raise doubts as to its ability to produce an appreciable degree of intravascular lysis. Alternatively, Singer's method may extract only a small fraction of the lysolecithin present in plasma, or may extract inhibitors along with it.

5.0, heating for 15 minutes in boiling water, filtering through paper, and neutralizing to pH 7.0 with 0.1 *N* NaOH. The removal of protein is very complete, and the inhibitory power of the filtrate for saponin and digitonin hemolysis is only from one-tenth to one-twentieth that of the original plasma. A variable and surprisingly large amount of lipoid, however, is carried down with the flocculated protein. Not realizing this in 1923, I thought that the result of this separation was evidence that the greater part of the inhibition is due to the plasma proteins, and it was not until recently that I found the parallelism between the inhibitory effect and protein content to be very incomplete (Ponder, 1943). By means of the same separation procedure, however, it can be shown that only about one-quarter to one-third of the total inhibition of saponin (or digitonin) hemolysis is due to plasma cholesterol. In a typical experiment, the inhibitory power of the plasma for saponin hemolysis is about 16 times that of the protein-free filtrate, and the amount of cholesterol in the diluted plasma is about 3 times that left in the filtrate. From these ratios, it appears that the plasma protein and the inhibitory substances other than cholesterol which are carried down with it are responsible for about 77 per cent of the total inhibition, while the cholesterol accounts for the remaining 23 per cent. In different experiments, the figure varies from 20 to 35 per cent.

Through the kindness of Dr. E. J. Cohn, Dr. H. B. Vickery, and Dr. Hans Neurath, I have obtained a number of plasma protein fractions in a state of known purity, and have measured their inhibitory power for different lysins. In each case the protein, dissolved in a phosphate buffer saline at pH 7.1, was introduced into a hemolytic system containing 0.8 ml. of lysin, 0.4 ml. of protein in known concentration, 0.4 ml. of saline, and 0.4 ml. of red cell suspension, and *R*-values were found in the usual way. The principal results are shown in Table III, which gives the *R*-values together with the concentrations of protein for which each was obtained.

It will be clear from Table III that none of the protein fractions has an inhibitory power of the same order as that of plasma itself. The most inhibitory preparation is the mixed globulin Fraction II + III, and this has only 10 to 15 per cent of the inhibitory power of whole plasma. The horse serum albumin and pseudoglobulin, the beef globulin, and the fibrinogen preparations exhibit their inhibitory effect, if any, in 1 per cent solution, and so their inhibitory power is less than 1 per cent of that of whole plasma. Since the sum of the inhibitory effects of the protein fractions does not amount to more than 25 per cent of the total inhibition,⁷ and since the cholesterol content ac-

⁷ It is quite likely that purification of the protein fractions alters their physical state in such a way that part of the inhibitory power is lost. Both Tsai and Lee and I have noticed that merely drying the plasma reduces its inhibitory power by 20 to 30 per cent, and Tsai and Lee attribute this to denaturation. Since some of the inhibitory effects appear to take place at the red cell surfaces rather than in the bulk

counts for only about 25 per cent, some 50 per cent of the inhibition has so far remained unaccounted for. This is also the conclusion reached by Tsai and Lee (1941).

The position has recently been greatly clarified by the investigations of Lee and Tsai (1942 *a, b*; and see also Tsai and Lee, 1941). They have approached the problem by studying the effect of cholesterol and lecithin sols on hemolysis by different lysins (saponin, digitonin, bile acids and salts, oleate, etc.), and have found that lecithin, although lytic *per se*, enhances the inhibitory effect of cholesterol 3 to 10 times in saponin systems. This enhancement depends on the value of the lecithin/cholesterol ratio as well as on the concentration of

TABLE III

Substance	Concentration	Saponin	Digitonin	Taurocholate	Oleate
	<i>gm. per cent</i>	<i>R</i>	<i>R</i>	<i>R</i>	<i>R</i>
Human serum albumin*	0.25	0.95	0.92	1.00	1.00
Human serum globulin fraction II + III†	0.02	1.11	1.14	1.12	1.20
Horse serum albumin§	1.00	0.95	1.35	1.80	1.45
Horse serum pseudoglobulin 	1.00	1.00	1.45	1.00	1.00
Human fibrinogen	1.00	1.10	1.17	1.14	1.20
Beef serum albumin¶	1.00	1.35	1.00	1.75	1.35

* Contained 1 in 1500 merthiolate, which gives $R = 0.92$ with digitonin. 97 per cent pure by electrophoresis, the balance being globulins.

† Also contained merthiolate. A mixture of gamma and beta globulins.

§ Electrophoretically homogeneous.

|| Electrophoretically homogeneous.

¶ Contained 4 per cent globulin impurities.

saponin, and seems to be due to an interaction between the lecithin, the cholesterol, and the saponin. A similar reinforcement is observed in systems containing digitonin or the bile salts. In addition to this, Lee and Tsai find that lecithin *per se* is inhibitory in saponin, digitonin, and crude bile acid systems, although not in systems containing the bile salts or the oleates. Their evidence is sufficient to lead to the conclusion that the 50 per cent of the

of the system it would not be surprising to find that small changes in physical characteristics would produce appreciable changes in inhibitory power. The process of purification, moreover, eliminates the enhancing effects of one component on another, and it is very doubtful if the sum of the inhibitions of the purified components of plasma, taken separately, would equal the inhibitory power of plasma itself. On the other hand, all the inhibitory substances acting together would probably produce a greater inhibition than that of whole plasma, in which some of the effect of inhibitors is offset by the opposed effects of accelerators.

inhibitory effect of plasma, which has so far remained unaccounted for, may represent the inhibitory effect of the plasma lecithin together with the enhancement which this substance confers on the cholesterol inhibition. The sum of the inhibitions produced by protein, cholesterol, and lecithin, acting singly, together with a generous allowance for enhancing effects, is thus enough to account for the whole inhibitory effect of plasma.⁸ The complete elucidation of the enhancing effects will probably turn out to be very difficult, and will have to take account of the fact that some of the lipoids and proteins of plasma are actually chemically combined (Bloor, 1943, p. 185).

2. "*X-Substance*," "*OBT Principle*," etc.—Farley (1939, 1942) and Horwitz

⁸ In connection with these inhibitory effects, Lee and Tsai have raised the old question as to whether the inhibition takes place in the bulk of the system, *i.e.* between inhibitor and lysin, or at the red cell surface. There are two distinct mechanisms by means of which inhibition can be produced. In the first, a reaction occurs between the lysin and the inhibitor, so that a quantity Δ of the lysin is rendered inert, the original concentration of lysin c_1 falling to a lower concentration c_2 , and Δ being equal to $c_1 - c_2$. In this case the added cells act merely as an indicator of the amount of free lysin. In the second, the inhibitor acts in the neighborhood of the cell surfaces, producing some kind of over-all effect which increases the resistance of the cells to the lysin R times, where $R = c_1/c_2$. These two mechanisms may be referred to as the Δ -mechanism and the R -mechanism respectively, and which of the two is operative in any given case requires to be decided on experimental grounds.

In the case of the inhibition of hemolysis (saponin, digitonin, crude bile acid, and natural hemolysin) by cholesterol sols, Lee and Tsai have shown that the inhibition increases with time and temperature when lysin and cholesterol react in the absence of red cells. This is good evidence for a Δ -mechanism. When cholesterol is added to lecithin, oleates, or the bile salts, and when lecithin is added to saponin, digitonin, crude bile acid, the bile salts, and the oleates, the inhibition observed when cells are added subsequently does not depend on the conditions of either time or temperature under which the cells and the inhibitor have stood together. Lee and Tsai propose that in these cases the inhibitors react with the lysins in a physical rather than in a chemical manner; there remains, however, the possibility that the inhibition is primarily due to an R -mechanism.

Both mechanisms are probably coexistent even in the simpler forms of inhibition. 80 per cent of the inhibition in arginine-saponin systems and 90 per cent of that in sucrose-saponin systems, is the result of an effect on the cells themselves, but the remainder may be the result of a reaction of the inhibitors with the lysin (Ponder, 1926). Yeager and I (1928) concluded that the inhibitory effect of the sugars is a double one both in saponin-sugar-cell systems and in taurocholate-sugar-cell systems, an R -mechanism being the more prominent in the case of the former, and a Δ -mechanism in the case of the latter. I have recently emphasized the effects of the plasma inhibitors at cell surfaces, or the R -mechanism (Ponder, 1943) partly because in the past I have treated this form of inhibition almost exclusively as a Δ -mechanism, or interaction between lysin and inhibitor. It seems likely, however, that both mechanisms are involved.

and Farley (1940) have described an inhibitor of digitonin hemolysis which they call "X-substance," or "OBT principle." This inhibitor is stated to be present in plasma in quantities which depend on the intake of thiamine or on the thiamine level, and Farley has proposed that the inhibitory effect of plasma on digitonin hemolysis be used as a method for thiamine assay. The inhibitory power of rabbit plasma is reported to be enormously increased by the daily administration of 100 mg. of thiamine subcutaneously for 7 days, plasma initially inhibitory in dilutions of about 1 in 2000 becoming detectably inhibitory in dilutions of about 1 in 1,000,000. Farley believes that the inhibitory substance is of low molecular weight, and related to, if not identical with, another inhibitory substance which he has obtained in crystalline form from tissues ("vitatropin").

I have been unable to confirm these results, either as regards the presence of a diffusible inhibitor or as regards the alleged effect of thiamine on the inhibitory power of serum on digitonin hemolysis. No measurable quantity of inhibitory substance diffuses from serum or plasma through either collodion or cellophane, and I have not obtained any significant increase in the inhibitory power of serum or plasma on digitonin or saponin hemolysis either in man or in the rabbit. In one experiment I gave each of 5 rabbits 100 mg. of thiamine chloride subcutaneously daily for a week. The inhibitory values for the sera, measured both by Farley's technique (1942) and by the method described in part 3 of this section, were substantially unchanged. In another experiment 6 persons received 50 mg. of subcutaneous thiamine daily for a week, and in 2 persons the injections were continued for 2 weeks. No changes in the inhibitory power of the sera were observed, other than the small differences which may occur from day to day in the untreated animal.

3. *Variations in the Inhibitory Power of Plasma.*—Very few investigations have been made on the variations in the inhibitory power of plasma. When investigating them, one may use saponin, digitonin, one of the bile salts, or any other lysin as the lysin to be inhibited, and the results found with one lysin need not necessarily be the same as those found with another. At this stage of our knowledge, however, we can suppose that a general idea of the variations in the power of inhibiting intravascular lysins will be provided by measurements of the power of inhibiting hemolysins such as saponin and digitonin *in vitro*. These lysins, being very stable, are more suitable for quantitative work than are the bile salts or the soaps.

A solution of digitonin⁹ (or saponin) is prepared, and decreasing amounts,

⁹ Dissolve 100 mg. of Merck's digitonin in 1000 ml. of saline. There seems to be some doubt about the solubility of this substance, which takes a considerable time to go into solution. The exact concentration is not important, but one wants it to be such that 1 ml., made up to 1.6 ml. with saline, gives complete lysis of 0.4 ml. of standard cell suspension in about 15 seconds. If lysis is more rapid, the stock solution can be diluted appropriately. It keeps well in the refrigerator.

1.2 ml., 1.1 ml., ... 0.5 ml., are added to a series of tubes. The volume of each is made up to 1.6 ml. with saline, and the tubes are placed in a water bath at 37°C.¹⁰ To each is added 0.4 ml. of a red cell suspension (the thrice washed cells of 1 ml. of human blood suspended in 20 ml. of saline) and the time for complete hemolysis is determined. The end point with digitonin and human cells is very sharp. Plotting the quantity of lysin used against the time required for complete lysis gives a time-concentration curve. This curve is not always the same for suspensions made from day to day from the blood of the same individual, the small variations observed apparently reflecting small variations in the resistance of the cells to digitonin, such as are also found in the resistance of cells to saponin hemolysis (see section IV).

The sera are obtained by drawing blood from the finger into Wright's tubes and centrifuging. Each serum is diluted 1 to 500 with saline (20 mm.³ to 10 ml.). To each of a series of tubes are added 1.2 ml., 1.1 ml., ... 0.5 ml. of digitonin, 0.4 ml. of the diluted serum, and enough saline to bring the volumes up to 1.6 ml. The tubes are allowed to stand for a measured time (usually 1 hour) at room temperature¹¹ (25°C.), and the time for complete lysis of 0.4 ml.

¹⁰ As in the case of saponin, the asymptotic concentration of digitonin required for complete hemolysis increases with increase in temperature. Thus at 37°C. complete lysis is brought about by 0.08 ml. of diluted stock solution of digitonin; at 30°C., 0.09 ml. is required, at 35°C., 0.10 ml., and at 40°C., 0.11 ml. The sharpness of the end point becomes gradually less as the temperature rises.

¹¹ The inhibition of digitonin hemolysis by serum and plasma increases with the length of time during which the lysin and the inhibitor are allowed to stand together before the completion of the hemolytic system by the addition of the cells. This seems to be the result of a reaction between cholesterol and other components of the plasma and the lysin. The time course of this reaction has been studied as a function of temperature by Lee and Tsai (1942 *b*) for systems containing a variety of lysins. They have found that the inhibitory action of cholesterol for saponin and digitonin hemolysis is increased by an increase in temperature, whereas that for bile salt and soap hemolysis is not; the inhibitory effect of lecithin is not affected by temperature either. The time course of the reaction between inhibitor and lysin is roughly exponential, being rapid at first and complete only after some hours. In the case of the inhibition produced by plasma, the temperature effect is small and the inhibition actually decreases with rise of temperature (Ponder, 1943). The time effects are also less conspicuous than in the case of inhibition by cholesterol sols, a plasma-digitonin system which gives an *R*-value of 1.37 when the plasma and the digitonin are allowed to stand together for 3 minutes before the cells are added giving an *R*-value of 1.43 when the reaction is allowed to go on for 30 minutes, and an *R*-value of 1.51 when it continues for 3 hours. It should not be concluded from this, however, that the inhibition is entirely due to a reaction between the inhibitor and the lysin in the bulk phase of the system (see footnote 8).

of cell suspension at 37°C. is then found. By comparing these times with those on the standard curve, a series of R -values are obtained in the usual way, and these, or their average, measure the inhibitory power of the serum.¹²

Proceeding in this way, we can make a number of observations.

(a) The inhibitory power of the serum of normal individuals varies from day to day, *e.g.* the following R -values were found for one person on 12 consecutive days: 1.39, 1.38, 1.35, 1.37, 1.29, 1.20, 1.24, 1.36, 1.39, 1.44, 1.30, and 1.22. There seems to be a cyclic character to the variations, the values decreasing for a few days and then increasing again. The variation in the R -value for the inhibitory plasma from 1.20 to 1.44 would have the same effect in the intact animal as a ± 10 per cent variation in the lysin concentration in the blood stream, and so would correspond to a variation of ± 10 per cent in the life of the average red cell.

(b) The inhibitory power of plasma is not highly correlated either with the cholesterol content or with the plasma protein content. In a series of 25 observations, the coefficient of correlation between the R -value and the cholesterol content was found to be 0.57 ± 0.14 , while that between the R -value

¹² The methods used by Farley for measuring inhibition are not at all satisfactory. He first finds the smallest quantity of digitonin required to hemolyze a standard suspension; this is done by taking 0.9, 0.8, . . . 0.1 ml. of digitonin of a certain concentration, adding saline and red cell suspension, and picking out the tube which contains the smallest amount of lysin which produces complete hemolysis after 5 minutes at 40°C. This amount clearly corresponds to a quantity a little greater than c_∞ , the asymptote of the digitonin time-dilution curve; but the extent to which it exceeds the asymptotic concentration depends on the position of the tube in the series, *e.g.* if there is lysis with 0.9 ml., but not with 0.8 ml., the tube selected contains about 9/8th of the asymptotic concentration, whereas if there is lysis with 0.2 ml. but not with 0.1 ml., the tube selected contains about double the asymptotic concentration. The quantity of lysin to be inhibited in the part of the experiment which follows may accordingly be variable, in terms of the asymptotic concentration, from experiment to experiment. To the quantity of lysin q selected as a result of this method of titration, plasma (or serum) diluted 1 in 1000, or more if necessary, is added in decreasing quantities 0.9 ml., 0.8 ml., . . . 0.1 ml. The smallest amount of plasma capable of inhibiting the quantity of lysin q is selected, and this measures the inhibitory power of the plasma; *e.g.*, inhibition produced in a dilution of 1 in 2000. What the method really measures is the amount of plasma required to inhibit a quantity of lysin ($q - c_\infty$), and the error of the method lies in the fact that the preliminary titration does not exactly define what ($q - c_\infty$) is. For example, if there is no lysis with 0.1 ml., but complete lysis with 0.2 ml., 0.2 ml. is selected as q ; then ($q - c_\infty$) is equal to 0.1. But so far as the titration employed is concerned, q might equally well be 0.19, or, for that matter, 0.11 ml., and ($q - c_\infty$) might really be even as small as 0.01. Such a tenfold difference in ($q - c_\infty$) would correspond to a tenfold difference in the amount of plasma required to produce the inhibition, and so the method is far from being an exact one.

and the plasma protein content was 0.55 ± 0.14 .¹³ This result is to be expected in view of Lee and Tsai's demonstration that the inhibitory effects are very complicated.

(c) Nevertheless, sharp changes in the cholesterol content or in the plasma protein content are usually associated with changes, in the same direction, in the *R*-value, e.g. an *R*-value of 1.33 was found on one day associated with a cholesterol content of 230 mg. per cent, and a fall in the cholesterol content to 138 mg. per cent on the following day was accompanied by a decrease in the *R*-value to 1.23 (the plasma protein content remaining constant at 7.0 gm. per cent). This associated variation would result from the cholesterol being responsible for part of the inhibitory power, but it may be contributed to by the same factors which bring about the change in the cholesterol content also bringing about a simultaneous change in the concentration of other inhibitors.

(d) Under conditions of poor nutrition and after major surgical operations, when the concentration of protein and cholesterol in the plasma is usually lowered, the inhibitory power of the plasma is very often reduced. I have already suggested that this reduction in inhibitory power, which would operate in the direction of favoring red cell destruction by intravascular lysins, may contribute to those postoperative anemias which are out of all proportion to the amount of blood lost (Seaman and Ponder, 1943).

IV. The Steady State and Hemolysis in Vivo

i

Suppose that the membrane of the least resistant red cell in a hemolytic system contains an amount of a component *S* and that the lysin acts irreversibly on *S* to convert it into another substance *S'*,



So long as a certain amount of *S* remains in the membrane, the semipermeability remains complete, but when a certain fraction of *S* is converted into *S'* the semipermeability is lost.¹⁴ Suppose that this loss occurs when *S'* has the

¹³ I have not been able to obtain as high a correlation between the cholesterol content of plasma and its inhibitory power as some investigators report. Tsai and Lee (1941) give a correlation coefficient of 0.922 for the inhibitory effect and the cholesterol content, and one of 0.267 for the inhibitory effect and the protein content. Yi and Meng (1941) also conclude that cholesterol is almost wholly responsible for the inhibitory effects. My experience agrees more closely with Lee and Tsai's later view (1942) that the total inhibitory effect of plasma is more complex than that produced by cholesterol alone.

¹⁴ For simplicity's sake, one can think of the lysin as digitonin, and as combining with the cholesterol *S* in the cell membrane to form cholesterol digitonide *S'*, but the situation is not as simple as this, and, except as an illustration, the idea is probably

particular value S'_0 ; the loss of semipermeability is the result of the breakdown of the membrane at its weakest or least resistant "key spots" (Ponder, 1941), and is followed by an all-or-none hemolysis. The transformation of S to S' corresponds to the utilization of a quantity of lysin x_0 , and the resistance of cell will be properly defined by this quantity x_0 . Another cell of greater resistance x_1 will not become semipermeable until a greater quantity of lysin x_1 is used up in forming compound S' in greater amount S'_1 ; a cell of still greater resistance will not become semipermeable until the still greater quantity x_2 of lysin has formed the still greater quantity of compound S'_2 , and so on. If there are N cells of varying resistance in the system, the distribution of the resistances, each measured in terms of the quantity of x necessary for the breakdown of semipermeability, will be described by some form of frequency distribution; *i.e.*, by

$$N = F(x) \quad (2)$$

In an *in vivo* hemolytic system a concentration of lysin c is maintained at a constant level, the lysin used up in reacting with S to form S' being constantly replaced by fresh lysin, and so the rate of utilization is constant and proportional to c , or

$$dx/dt = kc \quad (3)$$

and

$$t = x/kc, \quad \text{or} \quad x = kc \cdot t \quad (4)$$

Let the line AB in Fig. 1 represent the abscissa of the frequency distribution of the cells which are being added to the circulation from the hematopoietic tissues. The distribution itself must be imagined in a plane at right angles to the paper, a total number P being distributed into P_0 cells of least resistance x_0 , P_1 cells of resistance x_1 , . . . P_m cells of mean resistance x_m , . . . up to P_g cells of greatest resistance x_g . Alternatively we can think of the cells as being distributed according to the amount of the component S which each contains; thus the cells of resistance x_0 contain an amount of the component S_0 and hemolyze when this is all transformed into S' . And so on for the groups $x_1 \dots x_m \dots x_g$.

At $t = 0$, $x = 0$, but as t increases greater and greater quantities of lysin x combine with the cell component. The increase of x with t is represented along the line BC. Consider the group of resistance x_0 , starting when $x = 0$ at $t = 0$. As x increases with t , the component S_0 becomes transformed into S' ,

misleading. Many lytic substances have no affinity for cholesterol of the same sort as digitonin has; *i.e.*, do not form known compounds with it, and Tsai and Lee have remarked upon the fact that cholesterol appears to be inhibitory for lysins in general, largely irrespective of their chemical nature.

and the amount of S_0 which remains is always $(x_0 - x)$ until the quantity x_0 is combined at the time t_0 . Then $(x_0 - x_0) = 0$, and the cell hemolyzes. This is expressed in Fig. 1 by moving the cells of resistance x_0 along the line AD, parallel to the time axis, until they reach the line DC, on which the quantity of component transformed (x_0 on the axis BC) is equal to the quantity of component initially present (x_0 or S_0 on the axis AB). Beyond the line DC the cells cease to exist. In this case the length of the life of the cells of resistance x_0 in the blood stream will be t_0 .

Applying the same reasoning to the cells of group x_1 or to those of any other group, it will be apparent that the course of events is described by moving the

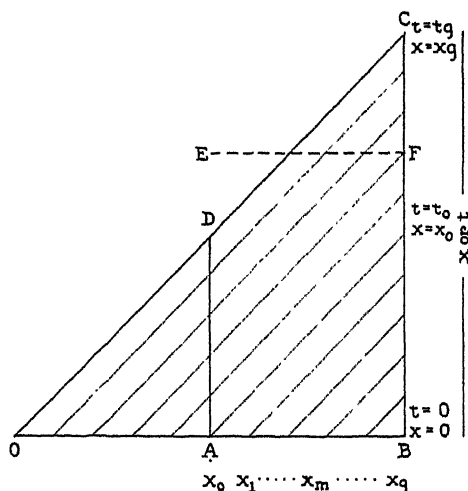


FIG. 1. For explanation, see text.

percentage distribution of P , erected on the line AB, along the line BC. As it moves, the distribution will at first remain intact, bounded on the one side by the line AD and by BC on the other. At time t_0 , however, x on BC will become equal to x_0 on OB, and the least resistant cells of the distribution, the group x_0 , will hemolyze. Next the group x_1 will hemolyze when $t = t_1$ and $x = x_1$ on BC, and so on for all the other groups $\dots x_m \dots x_g$. Thus as the moving distribution passes over the line DC, that part of it to the left or outside DC will be hemolyzed (*e.g.*, when the distribution has reached the position EF), and that part of it to the right or inside DC will be intact. Eventually all of the distribution will lie to the left of DC and even the most resistant cells x_g will have hemolyzed. Throughout, the length of the life in the blood stream of any group of cells is the distance from AB to DC, measured parallel to the time-axis; *i.e.*, the groups $x_0, x_1, \dots x_m, \dots x_g$ have life's $t_0, t_1, \dots t_m, \dots t_g$.

Now the solid enclosed as the frequency distribution P , originally at AB, moves over the surface ABCD will represent the total number of cells N in the circulation at any one time. The form of the frequency distribution of N can be found at any moment by appropriate sampling, experiment, and analysis (see Ponder, 1934, p. 161). In the steady state, a number of cells Q must be destroyed to balance the number P which is added in every interval of time, so $Q = P$, and the distribution of Q must be identical with the distribution of P . It will be clear, however, that in order to maintain a given distribution of the number N in the entire solid, the distributions of P and Q cannot be the same as that of N , for P must contain a greater proportion of the short lived elements and a smaller proportion of the long lived elements than N does. More specifically, if there are N_0 cells in the group x_0 in the distribution of N , the number P_0 of group x_0 in the distribution of P will be proportional to N_0/t_0 , t_0 being, as before, the life in the circulation of the cell of group x_0 . Similarly, $P_1 = N_1/t_1$, \dots $P_m = N_m/t_m$, \dots $P_g = N_g/t_g$.

Several conclusions now follow from these relations between the distributions N , P , and Q .

1. The distribution of new cells necessary for the maintenance of the population N in its steady state is more negatively skew than the distribution of the population N itself.

2. If we withdraw a sample of the population N and determine the resistance by adding a hemolysin, by reducing the tonicity of the medium, or by any other standard method, we find the resistances distributed according to the distribution $N = F(x)$ and the percentage hemolysis curves obtained in experiment are functions of $\int F(x) \cdot dx$. The resistance of any class of cell, however, cannot be interpreted as a measure of its age, although a relation between resistance and age is often stated or implied. The cells of least resistance x_0 , for example, contain the cells of resistance x_0 which have just entered the population, the cells originally of greater resistance x_1 which entered at time t_1 previously and whose life time in the blood stream has been t_1 , the cells of still greater resistance which entered at time t_2 previously and whose age is t_2 , and so on. The class of cells of greatest resistance, on the other hand, contains cells of resistance x_g , and these must necessarily be recent additions to the population; it is therefore true that the group of most resistant cells observed in fragility experiments is composed of the "youngest" cells, but equally "young" cells are to be found in any other resistance group, and the group of the most resistant cells observed in the experiment is certainly not composed of the "oldest" cells. The relation between resistance and age, in fact, is apparent only at the upper extreme of the frequency curve.

3. Since the distributions P and Q are more negatively skew than the distribution N , any sudden change in the rate of production or destruction will be reflected in the form of the S-shaped curve $N = \int F(x) \cdot dx$ which is obtained

by measuring resistance by the usual methods. These S-shaped curves are difficult to obtain in their entirety and in proper units, but the resistance of the group at the upper extreme is defined by the position of the asymptote of the time-dilution curve. Slight variations in the position of the asymptote, corresponding to variations in the balance between production and destruction, are accordingly to be expected if observations are made daily over long periods, and sufficiently detailed observations might even reveal a relation between variations in the position of the asymptote and the changes in the value of R described in section III, 3. The changes in the position of the asymptote will be familiar to anyone who has plotted time-dilution curves for suspensions of his own red cells daily over a considerable period of time. During the last 9 years I have noticed variations amounting to about ± 10 per cent in the position of the asymptote of time-dilution curves for saponin and suspensions of my own red cells, and in carrying out the experiments on inhibition of digitonin hemolysis described in section III, 3, I observed variations in the position of the asymptote of the digitonin time-dilution curve of about the same magnitude.

ii

Let us next consider how the total number of cells in the population N depends on the numbers in the population P and Q . N is the number of cells in the circulatory system, and there is a process producing new cells at a constant rate P . There is also another process removing the cells at a rate which must be proportional to N and which may also vary explicitly with the time t . Let the rate of removal be denoted by Nf , where f may be a function of t ; f may be called the "fractional rate of removal." The general problem is to find the value of N at any time t , given the initial value.¹⁵

The net rate of increase dN/dt is the difference between P , the rate of production, and Nf , the rate of removal, so

$$dN/dt = P - Nf \quad (5)$$

Let

$$\int f \cdot dt = F \quad (6)$$

where F is a function of t which may be found by integration when the function f is given.

The solution of the differential equation (5) is

$$N = (k + P \int e^{F} \cdot dt) \cdot e^{-F} \quad (7)$$

where k is a constant determined by the initial conditions. The value of this result is that it gives us the solution of the problem for any variation in the

¹⁵ I have to thank Dr. R. T. Cox for the general and special solutions in this section, as well as for the development of a satisfactory method of analyzing the experimental curves.

rate of fractional removal, provided that the two integrals $\int f \cdot dt$ and $\int e^F \cdot dt$ can be evaluated.

Special Solution 1: Constant Fractional Rate of Removal.—If the fractional rate of removal is constant, $f = a$, and

$$\int f \cdot dt = \int a \cdot dt = F = at \quad (8)$$

$$\int e^F \cdot dt = \int e^{at} \cdot dt = \frac{1}{a} \cdot e^{at} \quad (9)$$

Substituting in equation (7),

$$N = \left(k + \frac{P}{a} \cdot e^{at} \right) \cdot e^{-at} \quad (10)$$

When $t = 0$, let $N = N_0$; then $k = N_0 - P/a$, and so

$$N = \left(N_0 - \frac{P}{a} \right) \cdot e^{-at} + P/a \quad (11)$$

The first right-hand term approaches zero as t increases, and so N approaches a steady state with a value P/a . As the term does not change sign, if the value of N is initially above the steady state, $N_0 - P/a$ is positive and N never becomes less than P/a . Similarly if N has an initial value below the steady-state value, it never exceeds P/a . If N is initially equal to P/a , it never varies.

Special Solution 2: Uniformly Increasing or Decreasing Fractional Rate of Removal.—Let $f = a + bt$; then

$$\int f \cdot dt = \int (a + bt) \cdot dt = F = at + \frac{1}{2}bt^2 \quad (12)$$

The integral $\int e^F \cdot dt$ cannot be evaluated precisely, but the integration can be performed by regarding $e^{at + \frac{1}{2}bt^2}$ as an infinite series

$$1 + (at + \frac{1}{2}bt^2) + \frac{1}{2}(at + \frac{1}{2}bt^2)^2 + \frac{1}{6}(at + bt^2)^3 \dots$$

Expanding by the binomial theorem and collecting terms in each power of t , we get

$$\begin{aligned} e^{at + \frac{1}{2}bt^2} &= 1 + at + \frac{1}{2}(b + a^2)t^2 + \frac{1}{2}\left(ab + \frac{a^3}{3}\right)t^3 \\ &\quad + \frac{1}{4}\left(\frac{b^2}{2} + a^2b + \frac{a^4}{6}\right)t^4 + \dots \end{aligned}$$

Hence

$$\begin{aligned} \int e^F \cdot dt &= t + \frac{1}{2}at^2 + \frac{1}{6}(b + a^2)t^3 + \frac{1}{8}\left(ab + \frac{a^3}{3}\right)t^4 \\ &\quad + \frac{1}{20}\left(\frac{b^2}{2} + a^2b + \frac{a^4}{6}\right)t^5 + \dots \end{aligned} \quad (13)$$

This expression vanishes when $t = 0$; also, when $t = 0$, $e^{-F} = 1$. Referring to equation (7), we see that when $t = 0$, $N = k$, so N_0 may be written for k . Substituting in (7) the values of F and of $\int e^{-F} dt$ given by (12) and (13), we have

$$N = \left[N_0 + P \left\{ t + \frac{1}{2}at^2 + \frac{1}{6}(b + a^2)t^3 + \frac{1}{8}\left(ab + \frac{a^3}{3}\right)t^4 \dots \right\} \right] e^{-at - \frac{1}{2}bt^2} \quad (14)$$

Equations (11) and (14) can be combined to give the values of N in any case in which the graph of f against t is made up of straight segments. At any junction of two segments of the graph, the final value of N on the first segment is taken as the initial value N_0 on the second segment. A few examples follow.

Special Solution 3: Abrupt Transition between Two Constant Values of f .—Let f be constant for a long time at a value a' and then at a certain instant t_1 let it change instantaneously to a higher value a'' (see Fig. 2, A). At t_1 and for some time before we may assume that N is at its steady state value P/a' . When f suddenly assumes the new value a'' , equilibrium is destroyed and subsequent values of N are given by equation (11), the time t being reckoned from t_1 , the instant of transition, so that P/a' replaces N_0 and a'' replaces a' . The graph of N against t is shown in Fig. 2, B. The break in the slope of the graph corresponds to the change in dN/dt as f changes from a' to a'' .

Special Solution 4: Gradual Uniform Transition between Two Constant Values of f .—Let f be constant for a long time at a value a' . At a time t_1 let it start to increase linearly and let it attain a new value a'' at an instant t_2 and thereafter remain constant (Fig. 2, C). This case differs from the one just treated in that here dN/dt does not change discontinuously; consequently there will be no sharp break in the slope of the graph of N against t .

The value of N at t_1 and for some time previously is P/a' . Beginning at t_1 , equation (14) is used, with time reckoned from the instant t_1 as zero, P/a' replacing N_0 , a' replacing a , and $(a'' - a')/(t_2 - t_1)$ replacing b . This solution is valid until the instant t_2 , at which f becomes constant at the value a'' .¹⁶ The value found for N at t_2 now becomes N_0 in equation (11), in which the time is reckoned from the instant t_2 as zero and in which a'' replaces a . The form of the graph of N against t is shown in Fig. 2, D.

Special Solution 5: Linear Rise and Fall of f .—Let there be initially a steady state characterized by a constant value a' of f . At the instant t_1 let f start to rise uniformly, attaining a value a'' at the instant t_2 . Now let f start falling, and let it attain the value a' again at the instant t_3 , remaining constant thereafter. Let $t_3 - t_2 = t_2 - t_1$, the rate of fall being equal to the rate of rise.

¹⁶ The instant at which f assumes its new value often corresponds to a point of inflection on the experimental curve of N against t , but a point of inflection may occur under some circumstances while f is uniformly increasing.

In this case the solution up to the time t_2 is the same as that given in Fig. 2, D. From t_2 to t_3 the variation of N is given by equation (14) with time reckoned

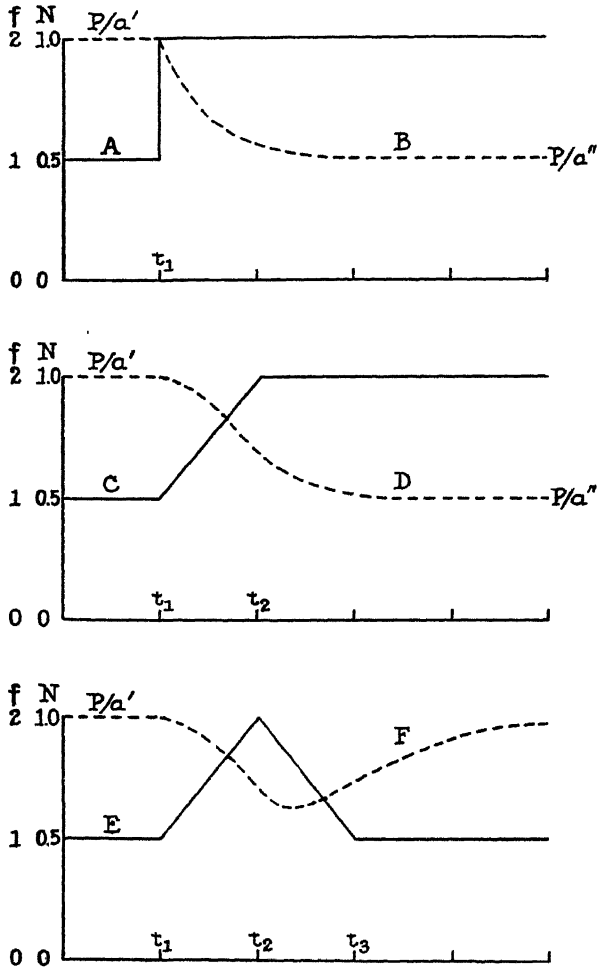


FIG. 2. Curves illustrative of special solutions 3, 4, and 5. Ordinates, N and f ; abscissa, time. Solid curves show variations in f ; dotted curves show variations in N . For explanation, see text.

from t_2 , with $b = -(a'' - a')/(t_3 - t_2)$, with a replaced by a'' , and with the value of N at t_2 being taken as N_0 . After t_3 , equation (11) is again used, with time reckoned from t_3 , so that N_0 is the value of N obtained at t_2 . Also a' replaces a . Graphs corresponding to this special solution are shown in Fig. 2, E and F.

The Analysis of Experimental Curves.—The general method of dealing with experimental curves which show the variation of N with time can best be illustrated by the analysis of a curve such as that shown in Fig. 3, which represents the changes in the red cell count observed during the first 15 days of the development of a hemolytic anemia due to the administration of phenylhydrazine. The method requires that somewhere the system passes from a changing state to a steady one, or to one so nearly steady that one can estimate

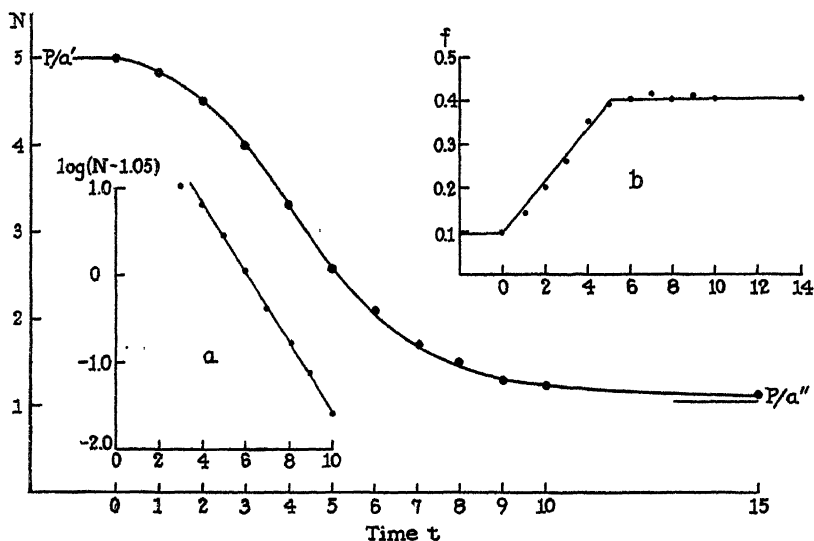


FIG. 3. Analysis of a curve for the fall in the red cell count N after administration of phenylhydrazine. See text for further description.

a steady-state value of N . In Fig. 3, a steady state seems to be attained at about 15 days, for which $N = 1.05$. Since in general $dN/dt = P - Nf$ (expression 5), and since $dN/dt = 0$ in the steady state, $N = P/f$, and so $P/f = 1.05$, f being the fractional rate of removal in the steady state. Now in the approach to this steady state, f must have been constant for some time, or else the steady state would never have been reached; and when f is constant, we can find N as a function of the time t . We have

$$dN/dt = P - Nf, \quad \text{or} \quad dN = -f(N - P/f) dt \quad (15)$$

Since P and f are constant, $d(P/f) = 0$, and we may replace dN by $d(N - P/f)$. So we have

$$-f \cdot dt = \frac{d(N - P/f)}{N - P/f} \quad (16)$$

which gives on integration

$$f(t_1 - t) = \log \frac{(N - P/f)_1}{N - P/f} \quad (17)$$

where t_1 is any specific value of the time t and $(N - P/f)_1$ is the value of $N - P/f$ at that instant. In the specific case of Fig. 3, we have $P/f = 1.05$, so

$$f(t_1 - t) = 2.3 \log_{10} \frac{(N - 1.05)_1}{N - 1.05}$$

We now plot $(N - 1.05)_1$ against t on semi-log paper, and see how far back on the curve f is constant (Fig. 3, inset *a*). From 5 to 10 days, one gets a good straight line, and the value of f turns out to be 0.41.

To find the value of f at times before 5 days, we first find P , and since $P/f = 1.05$ and $f = 0.41$, P must be 0.43. Now using expression (5) in the form

$$f = \frac{P - dN/dt}{N} \quad (18)$$

we draw tangents of slope dN/dt to the experimental curve. With values of N taken from the curve at the points where the tangents are drawn, and with the value 0.43 inserted for P in expression (18), the value of f can be found for any time t . Fig. 3, inset *b*, shows the way in which f varies with t ; for the first 4 days the fractional rate of removal rises almost linearly, and then becomes virtually constant at about 4 times its initial value.

Figs. 4 and 5 show two other examples of experimental curves and their analysis by this method. The data for Fig. 4 are taken from Ponder and Abels (1936), and show the fall in N following the injection of 80 mg. per kilo per day of quinine hydrochloride into a rabbit. In this very simple case, the fractional rate of removal seems almost immediately to assume its new value (see inset of Fig. 4). The data for Fig. 5 are taken from the paper by Dziemian (1942) on the effect of the injection of phenylhydrazine on the red cells of the rabbit, and show the result of a single injection. Here the value of f first rises and then falls to a level even lower than that for the normal steady state (see inset of Fig. 5). This probably means, in reality, that the increased rate of destruction has been followed by an increased rate of production.

iii

In the foregoing analysis, the total number of red cells in circulation is determined in terms of the rate of production P and the rate of destruction Q or Nf . If the process of destruction is an *in vivo* hemolytic process, some of the factors which control it can be specified further.

As expressed in equation (1), the rate of the fundamental lytic reaction by means of which the removal of cells is effected is proportional to c , and in the

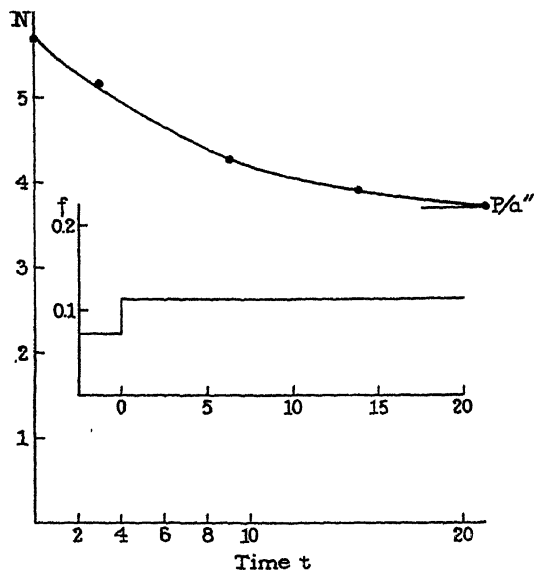


FIG. 4. Analysis of a curve for the fall in the red cell count following administration of quinine (data of Ponder and Abels, 1936). See text for further description.

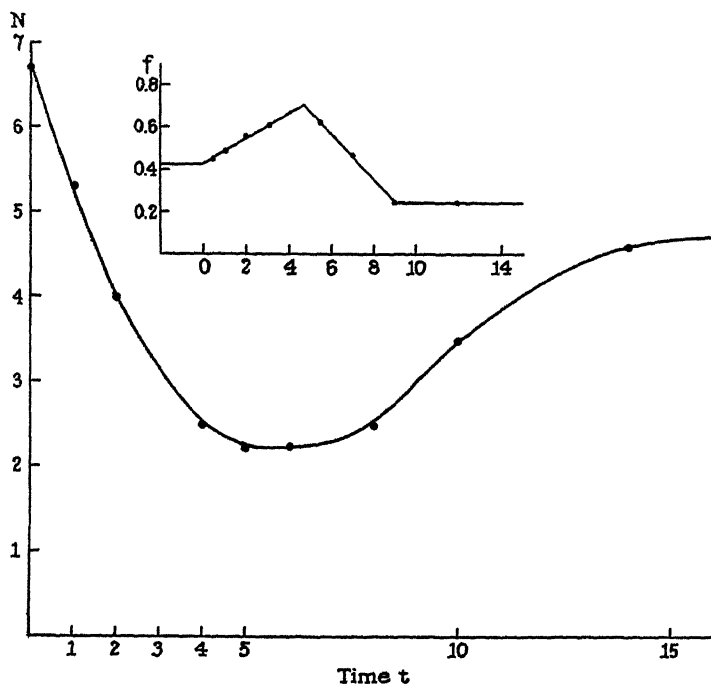


FIG. 5. Analysis of a curve for the changes in the red cell count after administration of phenylhydrazine (data of Dziemian, 1942). See text for further description.

steady state f has some special value (a in expression (8)) which makes the rate of red cell destruction equal to the rate of production. If c changes from one value c_0 to another c_1 , then $c_0/c_1 = R$, and f changes from a to a_1 , where $a_1 = a/R$. Such a change in the value of c can occur in two principal ways.

1. The rate of production of lysin may increase, as after a meal of fat, when the concentration of soaps in the blood stream rises. Many other instances of an increase in the concentration of intravascular lysins are known, particularly in pathological conditions.

2. Although the rate of production of lysin remains constant, the *effective* concentration of lysin in the blood stream may increase because of the addition of accelerators or the removal of inhibitors. In the normal steady state the concentration of lysin is kept at a certain effective value by the mixture of plasma accelerators and inhibitors, the net effect of which is an inhibitory one. For convenience, let us use the steady-state system as the standard one. If the net inhibitory power of the plasma decreases, so that R becomes less than unity with reference to the inhibitory power in the steady state, the effective concentration of lysin, which was c_0 originally, will increase to c_0/R . The net inhibitory power of the plasma may decrease, of course, either by the decrease in the quantity of the inhibitors or by an increase in the quantity of the accelerators, and in this way the effective concentration of lysin can undergo wide variations, especially in the direction of increasing.¹⁷ The way in which the net inhibition R_a depends on the individual inhibitions R_i and accelerations R_a has been discussed in the first section of this paper, and it is probably generally true that the inhibition or acceleration produced by any individual substance is roughly proportional to its quantity (see Ponder, 1939, 1941, 1943).

The analysis as outlined above requires in addition that P remains constant, and since this is a requirement which is not always fulfilled in actual experience, experimental curves should be analyzed in this way only when it is reasonable to believe that the change in N is due to changes in the extent of a destructive (usually hemolytic) process. For a more complete understanding of what occurs during the development of an anemia and recovery from it we would

¹⁷ It is very noticeable that there are many ways in which the red cell count can be reduced to a lower level, but only a few in which it can be raised above the normal steady state. Under no circumstances can it be raised very much, and in the few cases in which increases are observed they tend to be brought about at the expense of red cell size; *i.e.*, the quantity of reactive material, from the point of view of a hemolytic system, tends to remain constant. This suggests that there is a mechanism, not considered here, which becomes operative when the normal level is exceeded. *Decreases* in the concentration of intravascular lysins below the normal steady-state values, and *increases* in the net inhibitory power of the plasma above the normal steady-state values, either do not occur or are ineffective because the normal steady state constitutes a "ceiling state" in the physiology of the intact animal.

have to have some method of measuring variations in either *P* or *Q* independently. An indication, but not necessarily an exact measure, of variations in the latter would be provided by the urobilinogen excretion. This is certainly not a very satisfactory state of affairs; taken together with the great difficulty in extracting *in vivo* lysins quantitatively and the uncertainty with which the occurrences in complex hemolytic systems can be approached from the study of the effects of their isolated components, it probably means that the kinetics of *in vivo* hemolysis can be established in specially selected cases only.

SUMMARY

This paper is concerned with a variety of questions which bear on the occurrence of hemolysis *in vivo*, and with the possibility of regarding the contents of the blood stream as a hemolytic system in which a steady state is maintained by the production of new red cells to replace those which are destroyed. The material which is dealt with includes the following.

1. *Mixtures of Lysins, Accelerators, and Inhibitors*.—The effects of individual accelerators and inhibitors in mixtures, like the effects of individual lysins, are roughly additive in simple systems, the acceleration or inhibition produced by the individual substances being most conveniently measured in terms of *R*-values.

2. *Normal Intravascular Lysins*.—These probably play only a small part in red cell destruction unless their concentration rises to unusual levels, or unless their effects are enhanced by accelerators, or by the reduction of the concentration of normal inhibitors. The three normal *in vivo* hemolytic processes for which there is substantial evidence involve (a) the action of the bile salts and of the soaps derived from chyle, (b) the action of the spleen, and (c) the action of hemolytic substances derived from tissues. The recent observations of Maegraith, Findlay, and Martin on the presence of widely distributed tissue lysins are confirmed except for their conclusion that these lysins are species-specific. Species-specific tissue lysins, if present, are not the only lysins derivable from tissues by simple immersion in saline, for non-species-specific lytic substances can also be obtained, and seem to be similar to the "lysolecithin" which some regard as responsible for the action of the spleen on red cell fragility and shape.

3. *Plasma Inhibitors*.—About 30 per cent of the total inhibitory effect of plasma for saponin hemolysis is due to the contained cholesterol, while 25 per cent at most is due to the plasma proteins, particularly globulins. The remaining 45 per cent is probably accounted for by enhancing effects among the inhibitors; e.g., the enhancing effect of lecithin on the cholesterol inhibition. The mechanism of the inhibition is still incompletely understood; probably reactions between inhibitor and lysin and reactions between inhibitor and

components of the red cell surface are both involved, and it is important to observe that the inhibitory effect of plasma or of a plasma constituent may be greater in systems containing one lysin than in systems containing another. No evidence for diffusible inhibitory substances in plasma has been found, and the variations observed in the inhibitory power of human plasma seem to be related to the combined concentrations of cholesterol, protein, and probably lecithin, rather than to the cholesterol content alone. For this reason the inhibitory power tends to be low under conditions of poor nutrition.

4. *The Steady State and the Kinetics of Hemolysis In Vivo*.—On the assumption that the steady state is the result of a balance between a process which produces red cells and a process which destroys them, equations have been developed for the way in which cells of different resistances are affected when the rate of destruction changes. A method for analyzing experimental curves is described and illustrated. In general, this part of the paper relates the level of the red cell count in the animal to the intensity of the hemolytic processes taking place *in vivo*, and does not lend itself to detailed abstraction.

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THEORY AND MEASUREMENT OF VISUAL MECHANISMS

XII. ON VISUAL DUPLEXITY

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I

That the visual performance of vertebrates in general involves the separable functioning of two essentially different, morphologically distinguishable classes of retinal sensory units is a doctrine, the duplexity theory, which has developed from the suggestion of Schultze (1866). In outline, the proposition that retinal rods are concerned with visual responses at lower illuminations, cones with excitation at higher intensities,¹ is based upon correlations between retinal structure and the ethology of various animals, their nocturnal or diurnal activities; as well as upon the properties of photic excitability in different parts of the human retina.² These and developing related considerations, taken as a whole, led to the current very general view that retinal rods and their associated visual purple are connected with indistinct, colorless, scotopic vision, cones with distinct, colored, photopic vision. Thus, despite the sometimes considerable difficulty in the way of histological classification of photoreceptor units into two essential categories, even in a single retina, and certainly of regarding these as invariable, exclusive types in the vertebrate series,³ the

¹ von Kries, J., *Zur Theorie des Tages- und Dämmerungssehens*, in 1929, *Handbuch der normalen und pathologischen Physiologie*, (A. Bethe, G. von Bergmann, G. Embden, and A. Ellinger, editors), Berlin, Julius Springer, 12, pt. 1, 678. Arey, L. B., in 1932, *Special cytology*, (E. V. Cowdry, editor), New York, Paul B. Hoeber, Inc., 2nd edition, 3, 1213. Granit, R., 1936, *Die Elektrophysiologie der Netzhaut*. . . , Copenhagen. Hecht, S., 1937, *Physiol. Rev.*, 17, 239. von Studnitz, G., 1940, *Physiologie des Sehens Probleme der Biologie*, Leipzig, Akademische Verlagsgesellschaft, 3. Polyak, S., 1941, *The retina*, Chicago, University of Chicago Press. Walls, G. L., 1942, *The vertebrate eye and its adaptive radiation*, *Cranbrook Inst. Sc., Bull.*, 19, Bloomfield Hills, Michigan. Detwiler, S. R., 1943, *Vertebrate photoreceptors*, *Experimental Biology Monographs*, New York, Macmillan.

² Cf. Hecht, S., 1937, *Physiol. Rev.*, 17, 239; Crozier, W. J., and Wolf, E., 1941-42, *J. Gen. Physiol.*, 25, 369.

³ Cf. Tschermak, A., in 1929, *Handbuch der normalen und pathologischen Physiologie*, (A. Bethe, G. von Bergmann, G. Embden, and A. Ellinger, editors), Berlin, Julius Springer, 12, pt. 1, 295. Menner, E., 1928, *Z. vergleich. Physiol.*, 8, 761. Walls, G. L., 1934, *Am. J. Ophth.*, 17, 892. Verrier, M.-L., 1935, *Bull. biol. France et Belgique*, 20, suppl.; 1937, *Bull. biol. France et Belgique*, 71, 238; 1938, 78, 355.

ordinary assumption has come to be that the high-intensity and the low-intensity segments of duplex curves of visual performance represent respectively the quantitative properties of cones and of rods.

Despite the occurrence, in many forms, of more than simply two structural types of retinal receptor units, only in one case has the probable occurrence of three distinguishable parts to the visual performance contour been recognized.⁴ The phenomena of their photomechanical and otherwise experimentally induced movements,⁵ and of "spontaneous" movements,⁶ frequently serve additionally to separate the photoreceptor units into two distinct morphological categories, the histological types labelled rods and cones. In each such instance that has also been examined functionally a duplex performance contour (flicker; white light) has been found.⁷ Whereas, within the fovea of man and in forms with simplex retinas, only simplex performance contours (flicker) have been found.⁸ New illustrations are given in the present paper. In this sense, a basic feature of the duplexity rule is shown to be valid; that is, a retina obviously not simplex is correlated with a duplex (flicker) performance contour.⁹

We must be careful, however, not to proceed to the mistake of assuming that the quantitative properties of the scotopic and photopic portions of duplex contours are directly those of rods and of cones respectively. Even if they are determined at the retina, or are in simple proportion to retinal properties, the performance data represent at best properties of complex assemblages of units, as their analytical discussion demonstrates. Nor does it follow that any given criterion (*e.g.*, even color perception¹⁰) will necessarily be diagnostic of rod or

Walls, G. L., 1942, The vertebrate eye and its adaptive radiation, *Cranbrook Inst. Sc., Bull.*, **19**, Bloomfield Hills, Michigan. Walls, G. L., 1942, The visual cells and their history, in *Visual mechanisms*, (H. Klüver, editor). Biological symposia, **7**, Lancaster, The Jaques Cattell Press, 203.

⁴ Crozier, W. J., and Wolf, E., 1939-40, *J. Gen. Physiol.*, **23**, 667.

⁵ *E.g.* Laurens, H., and Detwiler, S. R., 1921, *J. Exp. Zool.*, **32**, 207; Verrier, M.-L., 1935, *Bull. Soc. zool. France*, **15**, 193.

⁶ Welsh, J. H., and Osborn, C. M., 1937, *J. Comp. Neurol.*, **66**, 349. Arcy, L. B., and Mundt, G. H., 1941, *Anat. Rec.*, **79**, suppl. 2, 5.

⁷ Wolf, E., and Zerrahn-Wolf, G., 1935-36, *J. Gen. Physiol.*, **19**, 495; Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1936-37, *J. Gen. Physiol.*, **20**, 211, 411; 1937-38, **21**, 17, 203, 313; 1938-39, **22**, 463; Crozier, W. J., and Wolf, E., 1939-40, **23**, 229, 667; 1943-44, **27**, 315.

⁸ Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1938, *Proc. Nat. Acad. Sc.*, **24**, 125, 216; Crozier, W. J., and Wolf, E. 1938, *Proc. Nat. Acad. Sc.*, **24**, 538; Crozier, W. J., Wolf, E., and Zerrahn-Wolf, G., 1938-39, *J. Gen. Physiol.*, **22**, 311, 555; 1939-40, **23**, 531; 1940-41, **24**, 317, 625; 1941-42, **25**, 369, 381; 1943-44, **27**, 119, 287.

⁹ 1938, *Proc. Nat. Acad. Sc.*, **24**, 125.

¹⁰ Cf. Wald, G., 1941, *J. Opt. Soc. America*, **31**, 461. Lewis, S. D., and Mandelbaum, J., 1943, *Arch. Ophth.*, **30**, 225.

cone functioning. What one is required to say is that, in duplex performance curves we have to do with the occurrence of two populations of neural effects in the constitution of the response contour.¹¹ This might well be found to occur in cases where only "cones" or only "rods" are revealed by ordinary histological inspection, but where either might really include more than one functional type. Reciprocally, it might easily happen that a structurally duplex retina should be associated with a simplex performance curve, but this we have not thusfar found. It is true that even within the fovea certain kinds of effects, involving intensity, image area, exposure-time, and wave-length, are not exactly "simplex";¹² but these questions are of a different sort. *We may also put to one side the details of interrelationships between "rod" effects and "cone" effects, in recognition of flicker for example,¹³ as revealed by the analytical dissection of compound contours, although it is to be noted that a proof is obtained that two independently modifiable groups of neural effects are represented in duplex contours. This is, however, an important part of the question as to the extent to which the "raw" data of performance as a function of intensity can be taken as a direct description of the properties of assemblages of neural units. The analytical form of the separated "rod" and "cone" performance contours is found to be of the same kind as when "rod" and "cone" properties can be dealt with quite independently, in the same retina¹⁴ or in different kinds of animals,¹⁵ and also in cases involving threshold discriminations where no integration of the two sets of effects may occur.¹⁶ These contours reflect, in their form and in the properties of their implicit parameters, the statistical attributes of effects due to assemblages of neural units.¹⁷ These properties are unequivocally not consistent with the view¹⁸ that the shapes of the performance contours as function of intensity reveal the organization of the physicochemical systems in rods and in cones which make excitation possible.¹⁹ Moreover, there is specific, quantitative evidence demonstrating that differ-

¹¹ 1938, *Proc. Nat. Acad. Sc.*, **24**, 538; 1938-39, *J. Gen. Physiol.*, **22**, 555.

¹² Cf. 1943-44, *J. Gen. Physiol.*, **27**, 119 (p. 122); Walters, H. V., and Wright, W. D., 1943, *Proc. Roy. Soc. London, Series B*, **131**, 340.

¹³ Cf. 1937-38, *J. Gen. Physiol.*, **21**, 17, 203, 313; 1938-39, **22**, 463; 1939-40, **23**, 229, 667, 677; 1940-41, **24**, 505, 635; 1941-42, **25**, 89, 293, 369; 1943-44, **27**, 287, 315, 401.

¹⁴ 1941-42, *J. Gen. Physiol.*, **25**, 369.

¹⁵ 1938, *Proc. Nat. Acad. Sc.*, **24**, 125, 538; 1938-39, *J. Gen. Physiol.*, **22**, 311, 451, 555; 1940-41, **24**, 317, 625; 1941-42, **25**, 381.

¹⁶ 1940, *Proc. Nat. Acad. Sc.*, **26**, 334, 382.

¹⁷ Cf. 1943-44, *J. Gen. Physiol.*, **27**, 119, 287, 401.

¹⁸ Hecht, S., 1937, *Physiol. Rev.*, **17**, 239; *Harvey Lectures*, 1937-38, **33**, 35.

¹⁹ 1937-38, *J. Gen. Physiol.*, **21**, 313, 463; 1938-39, **22**, 311, 487, 795; 1939, *Proc. Nat. Acad. Sc.*, **25**, 171; 1939-40, *J. Gen. Physiol.*, **23**, 143, 531; 1940-41, **24**, 505, 635; 1941-42, **25**, 89, 293, 369; 1943-44, **27**, 119, 287, 315.

ences in the shapes of "rod" and "cone" contours cannot be taken to signify differences in the physicochemical basis of excitation: the τ' constants (= abscissæ of inflection) of "rod" and "cone" components of duplex flicker contours in particular kinds of vertebrates are affected to the same extent by changes of temperature and of light-time fraction in the flash cycle.²⁰ Conversely, there is reasonable indication that differences in the forms of simplex contours, in a single animal, as a function of wave-length of light, cannot be taken to indicate differences in the photochemical basis of excitation.²¹ Nevertheless it is perfectly clear that the two groups of neural effects distinguishable in flicker contours for visually duplex vertebrates are organically distinct, although integratively related. They are separately modifiable by change of wave-length composition,²² retinal location,²³ subdivision of the image,²⁴ and by the introduction of the "pecten effect".²⁵ Their common quantitative dependence upon such variables as light-time fraction and temperature, already referred to, the quantitative character of the variation of performance in flicker recognition,²⁶ the properties of binocular simultaneous flicker,²⁷ all these are consistent with the conception that, although organically separable, the "rod" and "cone" populations of neural effects are intimately related in particular ways at the same general central nervous locus. Although the mass of this evidence is derived from the use of the only convenient method for testing visual performance quantitatively in a considerable variety of animals, there is no good reason either to ignore its import or to suppose that in general other modes of visual response would really be likely to give evidence of a very different meaning.

Thus while the parameters of the flicker contour have reproducible, specific significance in terms of the organization of the animals tested,²⁸ and must be understood to be determined by the statistical organization of the neural systems producing them, it does not follow that for quite different animals performance contours will necessarily be diagnostic of the type of peripheral receptors in the retina. Nor ought it be expected that there should be found a simple correlation between behavior, in general, and retinal histology. It is well known that while such a correlation is sometimes quite striking, it is often

²⁰ 1936-37, *J. Gen. Physiol.*, **20**, 411; 1940-41, **24**, 635.

²¹ 1943-44, *J. Gen. Physiol.*, **27**, 119.

²² 1941-42, *J. Gen. Physiol.*, **25**, 293; 1943-44, **27**, 119.

²³ 1941-42, *J. Gen. Physiol.*, **25**, 293; paper XIV, data to be published.

²⁴ 1943-44, *J. Gen. Physiol.*, **27**, 401.

²⁵ 1943-44, *J. Gen. Physiol.*, **27**, 287, 315.

²⁶ 1940-41, *J. Gen. Physiol.*, **24**, 505, 635; 1941-42, **25**, 293; 1943-44, **27**, 119.

²⁷ 1940-41, *J. Gen. Physiol.*, **24**, 505.

²⁸ 1937, *Proc. Nat. Acad. Sc.*, **23**, 516; 1938, **24**, 221; 1939, **25**, 171, 176, etc.

decidedly blurred.²⁹ We have already pointed out³⁰ that in essential respects (intensity range, slope constant) the flicker response contour for a nocturnal gecko (exclusively rod retina) can be practically identical with that for a sun-seeking turtle (with exclusively cone retina).³¹

We have sought to examine, in relation to these questions, flicker response contours from additional animal forms, several of which are here considered, with particular reference to the problem of correlations between visual performance and retinal organization. There are two chief points: (1) Is visual duplexity correlated with a retinally duplex structure, and (2) can quantitative performance properties in general be correlated with "rod" and "cone" categories of visual excitability? The examination of relevant data shows that the answer to (1) is, Yes; to (2), for an interestingly complex reason, the answer is, No.

II

Flicker contours were determined, by the method already described in detail,³² for the Florida "chameleon" *Anolis carolinensis* and for a soft-shelled turtle, *Trionyx (Amyda) emoryi* (Agassiz).³³ After completion of the observations, made in October and November, eyes of each were sectioned³⁴ and examined microscopically. The prediction from the nature of the flicker curves was that *Anolis* might have a simplex retina, *Trionyx* a duplex one;³⁵ this was found.

Anolis was studied after 45 minutes dark adaptation, using vigorously reactive individuals at air temperature $23.5^\circ \pm 1^\circ$. The responses to rotating stripes at the critical intensity involve a head motion up to 120° of turning in the direction of the movement of the stripes, then a quick "return." At $t_L = 0.10$ and 0.25 the response is less sharp than at higher values of t_L , particularly at lower intensities. At higher intensities the critical responses tend to be of smaller amplitude, and may even be

²⁹ Verrier, M.-L., 1932a, *Arch. zool. exp.*, **84**, 305; 1932b, *Compt. rend. Acad. sc.*, **195**, 1333; 1933c, *Bull. biol. France et Belgique*, **67**, 350. Detwiler, S. R.,¹ chapter V.

³⁰ 1938, *Proc. Nat. Acad. Sc.*, **24**, 538; 1938-39, *J. Gen. Physiol.*, **22**, 555.

³¹ For some reason which we do not understand Walls¹ speaks repeatedly of "the nocturnal *Pseudemys*;" statements about the retinas of such forms may be correct, but do not accord with the structural picture in our sections of the individuals we have used, nor with the habitual behavior we have described.

³² 1935-36, *J. Gen. Physiol.*, **19**, 495; 1936-37, **20**, 211; 1939-40, **23**, 531.

³³ We are obliged to Mr. A. Loveridge, of the Museum of Comparative Zoology, Harvard University, for the identification.

³⁴ Mr. D. M. Easton, to whom we are grateful, prepared these sections.

³⁵ Gillett, W. G., 1925, *Am. J. Physiol. Opt.*, **6**, 592, reported "only cones" in the retina of an *Amyda*.

reduced to a flickering movement of the eyeballs if the lizard has become comparatively rigid in posture; temporary reduction of I generally induced relaxation and freer responses under these conditions. The tests were made with three values of light-time fraction, $t_L = 0.10, 0.50, 0.90$.

TABLE I

Critical flash intensities (I_m) for response to flicker as a function of flash frequency F , at three values of the light-time fraction t_L , with *Anolis* ($22.8^\circ \pm 1^\circ$).

F per sec.	$t_L = 0.10$ $\log I_m \quad \log P.E.1$		$t_L = 0.50$ $\log I_m \quad \log P.E.1$		$t_L = 0.90$ $\log I_m \quad \log P.E.1$	
1					$\bar{2}.4655$	$\bar{3}.5222$
2			$\bar{3}.9429$	$\bar{4}.9821$	$\bar{2}.6959$	$\bar{3}.3039$
			$\bar{3}.9417$	$\bar{4}.1521$		
3			$\bar{2}.1538$	$\bar{4}.9364$		
			$\bar{2}.1644$	$\bar{4}.8206$		
5			$\bar{2}.3777$	$\bar{3}.0827$	$\bar{1}.1075$	$\bar{3}.8796$
					$\bar{1}.1137$	$\bar{2}.0231$
7			$\bar{2}.5501$	$\bar{3}.2579$	$\bar{1}.2749$	$\bar{3}.9239$
10	$\bar{2}.0515$	$\bar{4}.9714$	$\bar{2}.8617$	$\bar{3}.6556$	$\bar{1}.4291$	$\bar{3}.8316$
					$\bar{1}.4918$	$\bar{3}.9090$
15	$\bar{2}.3906$	$\bar{4}.8728$	$\bar{1}.0993$	$\bar{3}.5145$	$\bar{1}.8067$	$\bar{3}.5598$
20	$\bar{2}.6387$	$\bar{3}.1181$	$\bar{1}.2781$	$\bar{3}.6306$	0.0037	$\bar{2}.3935$
			$\bar{1}.2983$	$\bar{3}.8080$		
25	$\bar{2}.8857$	$\bar{3}.7382$	$\bar{1}.5183$	$\bar{2}.2085$	0.2477	$\bar{2}.7734$
30	$\bar{1}.0535$	$\bar{3}.7068$	$\bar{1}.7392$	$\bar{2}.0000$	0.4713	$\bar{2}.8093$
35	$\bar{1}.2054$	$\bar{3}.4550$	$\bar{1}.8958$	$\bar{2}.5120$	0.6172	$\bar{1}.0496$
40	$\bar{1}.4031$	$\bar{2}.1803$	0.1127	$\bar{2}.4913$	0.8389	$\bar{1}.1839$
43			0.3092	$\bar{2}.9763$		
45	$\bar{1}.7143$	$\bar{3}.8674$			1.1790	$\bar{1}.8796$
47			0.6145	$\bar{1}.1013$		
48	$\bar{1}.8893$	$\bar{2}.1495$			1.4584	0.3514
50	0.2151	$\bar{2}.3206$	0.9652	$\bar{1}.3636$	1.8731	0.1736
	0.1644	$\bar{2}.8153$				
52			1.1440	$\bar{1}.3734$	2.0734	0.2633
53	0.5591	$\bar{1}.1534$	1.2987	$\bar{1}.8796$		
	0.4465	$\bar{1}.0039$				
54			1.5290	$\bar{1}.8517$		
55	1.2100	$\bar{1}.9612$	1.9777	0.3132		
	0.8157	$\bar{1}.7814$				
57	1.9848	$\bar{1}.8887$				

The averages of critical flash intensities entered in Table I are derived from three measurements with each of the same five individuals throughout. As shown in Fig. 1, each contour is accurately described by a probability summation. The curve moves to higher intensities as t_L is increased, in the usual way, and $F_{max.}$ is lowered. Fig. 2 shows that the parameter $\sigma'_{\log I}$ is constant, since on the probability grid the lines through the data are parallel. These

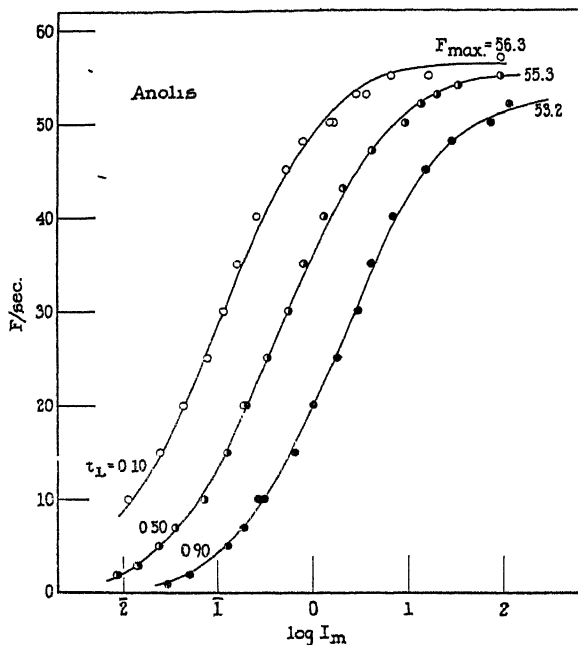


FIG. 1. Flicker contours for *Anolis*, with three light-time fractions. Data in Table I. The curves are probability integrals (cf. Fig. 2), calculated to the asymptotic maxima indicated.

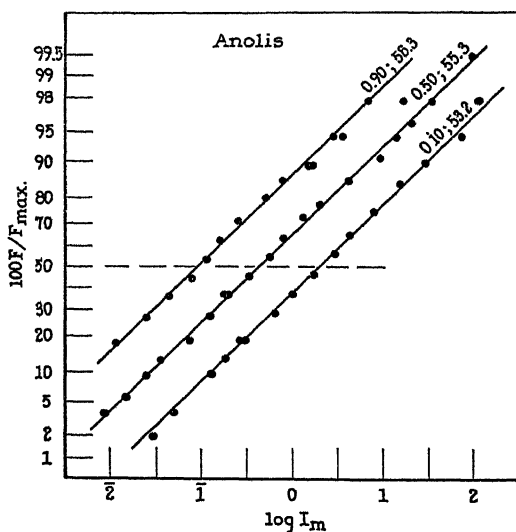


FIG. 2. Flicker response data for *Anolis* (Fig. 1) shown on a probability grid. The slopes for the three light-time fractions, and hence $\sigma'_{\log I}$, are the same.

relations are those found in other cases examined.²⁰ The relation of F_{max} and of τ' , the abscissa of inflection, to t_L is rectilinear, as shown in Figs. 4 and 5.

The statistically constant relative variation of I_1 in the *Anolis* data is a little higher (N is smaller) than in our series with fishes,³⁶ gecko,³⁰ crayfish,³⁷ *Anax*,³⁸ about the same as that with *Pseudemys*,³⁹ but lower than for *Phrynosoma*,⁴⁰ the mean value of $P.E._1/I_m$ is 0.0316. The internal correlation of the meas-

TABLE II

Critical flash intensities (I_m) for response to flicker as a function of flash frequency F , at three values of the light-time fraction t_L , with *Trionyx* ($22.6^\circ \pm 1^\circ$).

F per sec.	$t_L = 0.10$ $\log I_m$ $\log P.E._1$		$t_L = 0.50$ $\log I_m$ $\log P.E._1$		$t_L = 0.90$ $\log I_m$ $\log P.E._1$	
2	4.8844	5.5831	3.6083	4.2887	2.2720	4.8907
3	3.2417	5.8061	2.0241	4.6254	2.7162	3.0648
4	3.4352	4.0172				
5	3.6501	4.2936	2.4355	4.8949	1.1473	3.5463
8	2.0675	4.2587	2.8074	3.0857	1.5519	3.3946
10	2.2900	4.9139	1.0103	3.6247	1.7358	3.6079
15	2.5898	4.7084	1.2983	3.6729	0.0342	2.3467
20	2.7747	3.1361	1.4946	2.0188	0.2274	2.5485
25	1.0174	3.5856	1.7448	2.2618	0.4729	2.8739
30	1.2475	3.3167	1.9566	2.2294	0.6710	2.9050
35	1.4480	2.0457	0.1626	2.7938	0.8818	1.3046
40	1.7483	2.0696	0.4630	2.4198	1.1903	1.7475
45	0.0799	2.6477	0.8257	1.2708	1.6036	1.7672
	(0.1377	2.7329)				
48	0.3109	2.3394	1.2001	1.3320	1.9931	0.2065
	(0.4866	2.8552)				
50	0.6974	1.2820	1.5469	1.8507		
	(0.9245	1.5461)				
51	1.1418	1.4465				
	(1.4544	1.7699)				

urements²⁴ (among the *individuals*, here) is not too low (0.568 to 0.738) and r is maximum at $t_L = 0.50$, as found for other instances.²⁴

The data on *Trionyx* are given in Table II.

These animals, young, kept in a shallow tank with sand and gravel, and fed with *Enchytraeus* and lettuce, were observed to be almost constantly buried in the mud

³⁶ 1937-38, *J. Gen. Physiol.*, **21**, 17; 1938-39, **22**, 463, etc.

³⁷ 1939-40, *J. Gen. Physiol.*, **23**, 1.

³⁸ 1936-37, *J. Gen. Physiol.*, **20**, 363, 393; 1937-38, **21**, 223, 463; 1938-39, **22**, 795.

³⁹ 1938-39, *J. Gen. Physiol.*, **22**, 311, 1939-40, **23**, 531.

⁴⁰ 1940-41, *J. Gen. Physiol.*, **24**, 317.

during the daytime. The same five individuals were used throughout, with 45 minutes preliminary dark adaptation. Each turtle was used with just a little water in its cylindrical container, the head protruding into air. The critical end-points were obtained from head nystagmus. In *Trionyx* these movements are less sharp, slower, and less extensive than in (young) *Pseudemys*.

Particular care was taken in the work at lower flash frequencies. The data show at these lower frequencies (Fig. 3) a persistent "bump." This is not

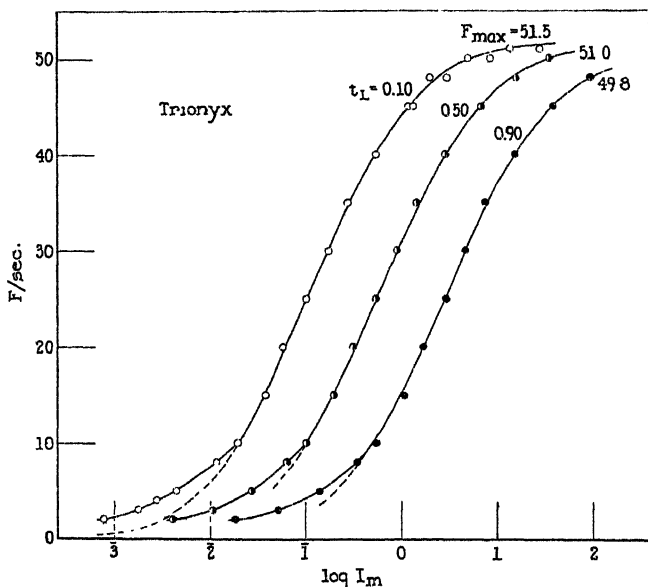


FIG. 3. Flicker contours for *Trionyx*, with three light-time fractions. Data in Table II. Above $F = 10$ the curves drawn are probability integrals calculated to the maxima indicated and with the same $\sigma'_{\log I}$. The departures below $F = 10$ are interpreted as due to a second group of excited neural units ("rods"). For $t_L = 0.10$ the difference curve as obtained in the usual way has been drawn. See text.

similar in shape to that found for the $F - \log I$ curve in the gecko.³⁰ It was not possible to secure responses below $F = 2$, but the behavior of this "tail" at the several light-times shows the "tail" to be not very probably due to iris (as in gecko case). Treated by the method of dissection⁴¹ the presumptive "rod" part is larger the greater t_L is.

As with frog⁴² and *Triturus*,⁴ the presumptive rod contribution to the $F -$

⁴¹ 1937-38, *J. Gen. Physiol.*, **21**, 17; 1938-39, **22**, 463; 1939-40, **23**, 677; 1940-41, **24**, 505, 635; 1941-42, **25**, 293, 369; 1943-44, **27**, 119.

⁴² 1939-40, *J. Gen. Physiol.*, **23**, 229.

log I contour in Fig. 3 is small. Subsequent histological examination of the eyes of these *Trionyx* showed only a comparatively few rod units; but in our frogs, and in certain fishes we have used,³⁶ the "rod" component of the flicker contour is also small although (relative to numbers of cones) the proportion of retinal rods is high.

It should be noticed here that, as we have already mentioned for other cases,⁸ the comparison of visual excitabilities by a chosen technique may lead to essentially meaningless results if a particular set of arbitrary standard conditions is imposed. Suppose, for example, that one desired to compare the capacities of *Anolis* and *Trionyx* to resolve stripe images. It is clear, from Fig. 6 for exam-

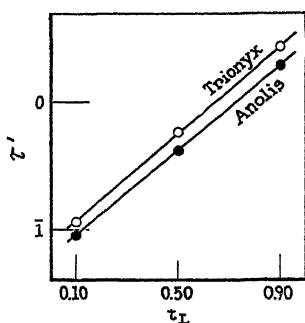


FIG. 4

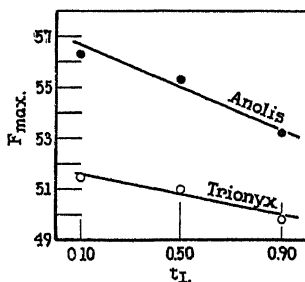


FIG. 5

FIG. 4. For *Trionyx* and for *Anolis* the abscissa of inflection (τ') of the flicker contour is directly proportional to the light-time fraction t_L , and the curves are shifted to essentially the same extent when t_L is changed.

FIG. 5. F_{max} . (Figs. 1 and 2) declines with t_L for both *Anolis* and *Trionyx*.

ple that the answer would depend upon the intensity level at which one might chose to operate. This type of procedure has been rather extensively used, as it happens,⁴³ and although we have already commented upon the essential point involved³⁶ it is worth re-emphasizing that unambiguous statements of visual properties can be achieved only in terms of properties of formulations of functional dependence.⁴⁴ It is part of the difficulty and fascination of this problem that the achievement of such statements must cope with the multivariate character of visual "thresholds."

Fig. 3 shows that the *Trionyx* "cone" curves are very similar to those for *Anolis* (Fig. 1), although with lower F_{max} . and situated at slightly higher intensities. Fig. 4 indicates that with increase of t_L the abscissa of inflection τ'

⁴³ Cf. Smith, K. U., 1938, *Psychol. Bull.*, **35**, 193; Kalmus, H., 1943, *J. Genetics*, **45**, 206.

⁴⁴ 1938, *Proc. Nat. Acad. Sc.*, **24**, 542; 1939, **25**, 171, 176; 1940-41, *J. Gen. Physiol.*, **25**, 89.

changes at just about the same rate as for *Anolis* (and *Phrynosoma*), although (Fig. 5) F_{max} declines less rapidly.

In line with the sharper quality, on the whole, of its responses at critical intensity, we find that for *Trionyx* the variation of I_r (cf. Table II) is a little less than for *Anolis*, $P.E._1/I_1$ averaging 0.025. The internal correlation evident in the measurements is also higher, since r in the expression $\sigma_s = (\bar{\sigma}/\sqrt{2})(\sqrt{1-r})$ declines only from 0.895 ($t_L = 0.10$) to 0.780 ($t_L = 0.90$).

Sections of the eye of *Anolis* disclose conditions paralleling those apparently to be taken as typical for eyes of lacertilians. Only one kind of retinal unit is recognizable under careful scrutiny, the cone. There is a deep, well marked foveal depression; in this part the density of cones is four to five times as great as in the peripheral retina. There is present a pecten, in the form of a simple, finger-like, protrusion based at the entrance of the optic nerve and extending toward the lens; this structure is deeply pigmented; it is quite unlikely that the foveal region can be covered by the shadow image of this pecten.

The retina of *Trionyx* shows mostly stout cones;³⁶ in the foveal region the number per unit area is slightly higher than elsewhere. Rods are sparsely intermingled with these cones. They are relatively robust and are well extended in dark adapted retinas. In the relatively flat, thinned foveal region and elsewhere in the retina the proportion of rods to cones is at best 1:5, at poorest 1:8.

III

Fig. 6 compares "cone" flicker contours, $t_L = 0.50$, for *Anolis*, *Trionyx*, *Phrynosoma*,⁴⁰ *Pseudemys*,³⁹ *Taeniopygia*,⁴⁵ and *Passer*.⁴⁶ The curves for the two lizards, *Phrynosoma* and *Anolis*, are of a similar steepness despite the difference in maximum F . The curves for the two (closely related) birds are not unlike. The curves for the two turtles, *Pseudemys* and *Trionyx*, are not at all similar. The slight temperature differences obtaining in the data on turtles and lizards we can for present purposes ignore; and since we know⁴⁷ that change of temperature does not affect either F_{max} or the shape constant of the curve ($\sigma'_{\log I}$), at any level of t_L (0.10 to 0.90), we can use the form constants $\sigma'_{\log I}$ as basis for attempted classification of the contours. This is the best invariant measure for the purpose, since the extent of change of F_{max} with t_L ,⁴⁸ and of the abscissa of inflection r' with t_L ,⁴⁸ is characteristic of the animal; hence no "standard conditions," as regards t_L , can be chosen to give non-arbitrary values

⁴⁵ 1940-41, *J. Gen. Physiol.*, **24**, 625; 1941-42, **25**, 381; 1943-44, **27**, 287.

⁴⁶ 1943-44, *J. Gen. Physiol.*, **27**, 315.

⁴⁷ 1936-37, *J. Gen. Physiol.*, **20**, 393, 411; 1938, *Proc. Nat. Acad. Sc.*, **24**, 216; 1938-39, *J. Gen. Physiol.*, **22**, 311; 1939-40, **23**, 531.

⁴⁸ 1936-37, *J. Gen. Physiol.*, **20**, 393, 411; 1937-38, **21**, 463, 1938-39, **22**, 311, 795.

of F_{max} , or τ' for purposes of classification of the curves. Whereas $\sigma'_{\log I}$, the standard deviation of the first derivative of F vs. $\log I$ with $F_{max} = 100$, is only slightly affected by image area (in a given eye⁴⁹) or by wave-length,⁵⁰ it is independent of t_L and of temperature, although sharply influenced by subdivision of the image⁵¹ and in birds by the influence of the pecten⁵² (for high values of t_L , with the moving stripe technique).

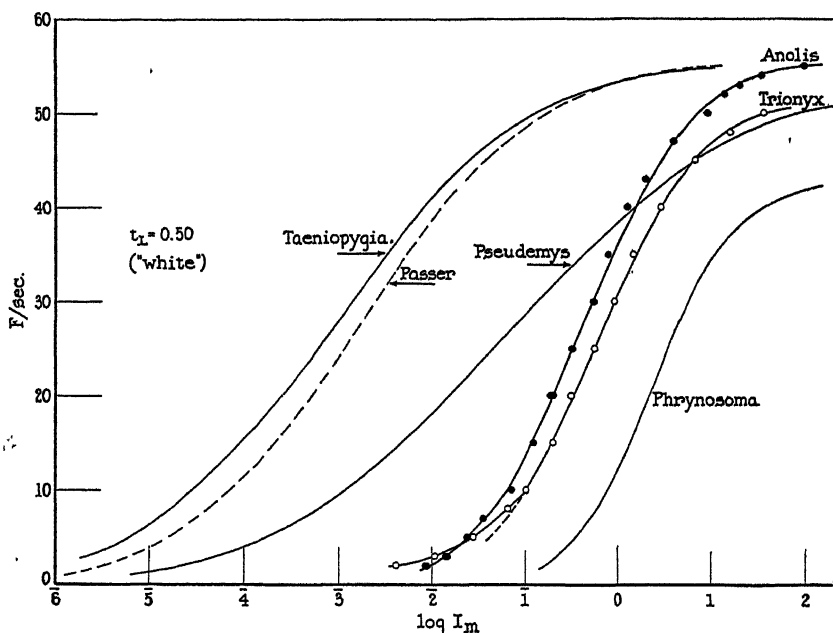


FIG. 6. Flicker contours at $t_L = 0.50$ are shown for the lizard *Anolis* and for the turtle *Trionyx*, together with contours under the same conditions previously obtained for *Phrynosoma* (lizard), *Pseudemys* (turtle), and for the birds *Taeniopygia* and *Passer*; these are all "cone" curves. See text.

On a probability grid (Fig. 7) curves in Fig. 6 have been drawn, together with certain others from earlier reports. All are for $t_L = 0.50$, "white" light, and are "cone" contours except for *Sphaerodactylus* ("rod").¹¹ It is pretty clear that these contours fall into two fairly distinct groups. The curves for *Xiphophorus*,³⁶ hybrids of *Xiphophorus* with *Platypoecilus*,³⁶ *Enneacanthus*,³⁶ *Fundulus*,⁵³ *Rana*,⁴² *Triturus*,⁴ *Sphaerodactylus*,³⁰ *Pseudemys*,³⁹ *Taeniopygia*,⁵²

⁴⁹ 1937-38, *J. Gen. Physiol.*, **21**, 223; and a following paper.

⁵⁰ 1941-42, *J. Gen. Physiol.*, **25**, 293, 381; 1943-44, **27**, 119.

⁵¹ 1943-44, *J. Gen. Physiol.*, **27**, 401.

⁵² 1943-44, *J. Gen. Physiol.*, **27**, 287, 315.

⁵³ 1939-40, *J. Gen. Physiol.*, **23**, 677.

*Passer*⁵² (cf. Fig. 6), and man, are of lower slope and lower τ' . Those for *Trionyx*, *Phrynosoma*, and *Anolis* are steeper, with higher τ' . It is true that the *Fundulus* slope might be taken as intermediate, but calculation from all available contours shows that, including those (e.g., man,⁵⁴ ape,⁵⁵ bee⁵⁶) obtained by different procedures but with "white" light and large image fields, $\sigma'_{\log I}$ for "cone" sections ranges from 0.72 to 2.3, with definite modes at ca. 0.80 and at

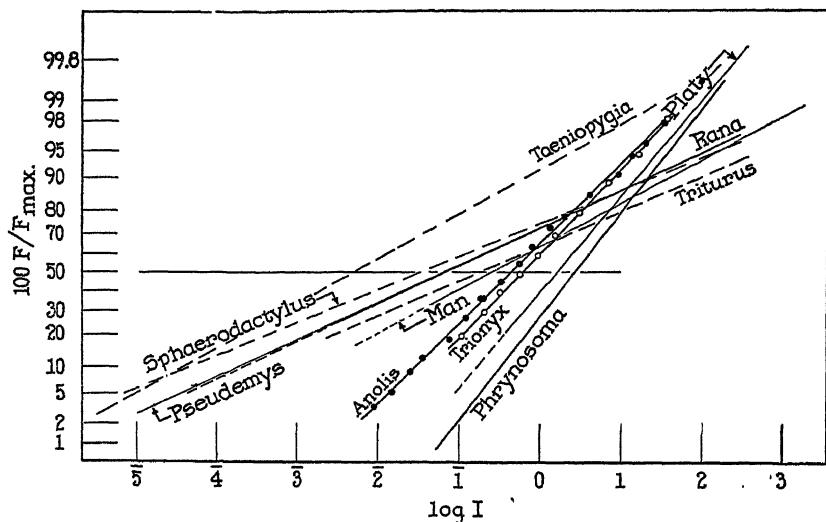


FIG. 7. Flicker contours, $t_L = 50$, white light, rotating cylinder technique, for various animals as indicated; on a probability grid. The data for all but *Anolis* and *Trionyx* are taken from previous papers. For man, *Rana*, *Triturus*, and *Platy* the "cone" curves of duplex contours are given; the other curves are simplex. "Cone" curves for several other fishes are not too dissimilar to that for *Platy*, and the "cone" curve from the duplex curve for the sparrow *Passer* is very similar in slope to that for the finch *Taeniopygia*; these latter are not given, to avoid crowding the figure. It is apparent that these curves fall into two rather sharply contrasted groups; see text.

17.5, no cases at 13.5. This distribution is based on 30 contours and sets of contours, but it is not essentially disturbed if account is taken of other material in the literature¹⁸ and of the effects of image area and retinal position;⁵⁷ 14 cases fall in the steeper group, 16 in the other. While a larger assemblage of vertebrates might show intermediates, this is the state of present information.

It is striking that although the two fringillids go together in this classifica-

⁵⁴ 1937-38, *J. Gen. Physiol.*, **21**, 203; 1940-41, **24**, 505, 635; 1941-42, **25**, 293.

⁵⁵ Cf. Brecher, G. A., 1935, *Z. vergleich. Physiol.*, **22**, 539.

⁵⁶ 1935-36, *J. Gen. Physiol.*, **19**, 503, 1937-38, **21**, 223; 1938-39, **22**, 451.

⁵⁷ Data in a following paper.

tion, as do the two lizards *Anolis* and *Phrynosoma* and the amphibians *Rana* and *Triturus*, and the various fishes, the straight zoological ordering of the curves is plainly impossible: the turtles *Pseudemys* and *Trionyx* are respectively in the less steep and in the steeper groups; the contrast between gecko and lizards is striking; the "rod" curve of the gecko is in the less steep group. We shall point out that all other rod flicker contours known (from visually duplex animals) give slopes agreeing precisely (in mode and range) with those of our steeper cone group, and also that $\sigma'_{\log I}$ for a cone contour, by simple change of conditions, can be caused to shift from one group to the other (man,^{24,52} birds⁵²). The only directly relevant evidence⁵⁸ shows that genetic constitution can determine $\sigma'_{\log I}$ in the absence of such effects. Yet it is to be noted that for such different arthropods as *Apis*, *Anax*, *Asellus*⁵⁹ $\sigma'_{\log I}$ is practically identical (0.96), almost the same as for our modal steep "cone" curves (and our "rod" curves in duplex contours), whereas for *Cambarus*⁶⁰ the value is notably higher (1.41), and for *Uca* also in all probability.⁶¹

If we turn to the "rod" components of duplex contours (Fig. 8) we find them to be of reasonably uniform high slope; 14 examples, from data already published, give a mean $\sigma'_{\log I}$ of 0.814, ranging from 0.50 to 1.104, in fishes, birds, and man. This value is slightly influenced by image area, retinal position, wave-length composition, and subdivision of the image. In man the values for the "rod" segment as isolated by the use of subdivided fields or other arrangements,^{51,52} although increased slightly over their values when cone competition is not present, are not changed markedly,—the highest estimates for $\sigma'_{\log I}$ being 0.75 to 0.96 for these "pure rod" populations.

It might be tempting to argue that one of the "cone" groups corresponds in some significant way to the essential "rod" group, since their modal values of $\sigma'_{\log I}$ are in excellent agreement. "Rod" and "cone" differences not dissimilar appear in the properties of other visual performance contours (man).⁶² But it is a simple matter experimentally to convert a cone curve of the lower slope group into the upper, as by subdivision of the flickered field into several parts a cone contour with $\sigma'_{\log I} = 1.81$ can be made steeper until $\sigma'_{\log I} = 1.25$ or 0.82; in the retinal periphery, using a small image, $\sigma'_{\log I}$ can be changed from 1.85 to 1.11 by shifting from blue light to red.⁶³ Employing the method of rotated stripes with fixed inclined opaque bars in the field ("pecten effect") $\sigma'_{\log I}$ can be reduced from 2.10 (or 1.65) to 0.82 by reducing the dark-time in the flash

⁵⁸ 1937–38, *J. Gen. Physiol.*, **21**, 17; 1938–39, **22**, 463; 1939–40, **23**, 143. 1937, *Proc. Nat. Acad. Sc.*, **23**, 516; 1938, **24**, 221, 542; 1939, **25**, 176.

⁵⁹ 1938–39, *J. Gen. Physiol.*, **22**, 451.

⁶⁰ 1939–40, *J. Gen. Physiol.*, **23**, 1.

⁶¹ 1937–38, *J. Gen. Physiol.*, **21**, 223.

⁶² 1940, *Proc. Nat. Acad. Sc.*, **26**, 334, 382.

⁶³ 1941–42, *J. Gen. Physiol.*, **25**, 293.

cycle. In birds this occurs naturally,⁵² with reduction of $\sigma'_{\log I}$ from 1.65–1.68 to 1.16, and in all probability could be pushed further. In Fig. 7 certain of these effects are illustrated. It should be noted that there is (Figs. 6 and 7) no necessary correlation of $\sigma'_{\log I}$ with the magnitude of τ' .

Thus in the diverse "cone" flicker contours the most nearly invariant index ($\sigma'_{\log I}$) of quantitative performance characteristics falls into two rather distinct

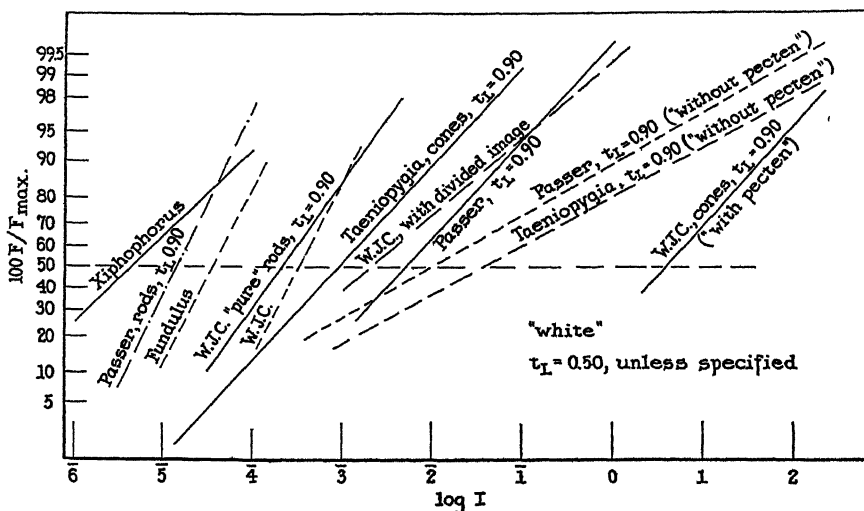


FIG. 8. Various presumptively "rod" flicker contours, white light as obtained with different animals are shown on a probability grid. These include data from *Xiphophorus*, *Fundulus*, *Passer*, and man. The slopes for other "rod" contours are included within the range of slopes illustrated. It is notable that these slopes, even when obtained under such conditions as not to involve cone complication, are of the type found for one of the two groups of cone contours in Fig. 7. The occurrence of the "pecten effect" serves to produce in *Taeniopygia*, *Passer*, and man, a steepening of the "cone" contours, from the slope indicated by the *computed* curves to that characteristic of the "rod" contours in duplex animals (or of the other group of "cone" effects in Fig. 7). See text.

groups, and is only slightly influenced in magnitude by such variables as image area; one of these groups of slope constants agrees precisely with that obtained from "rod" segments of duplex contours; yet a "cone" curve of one group can be caused, by one of several simple methods (subdivision of the image, or change of spectral zone; or, in birds, reduction of the percentage dark-time) to assume the slope constant typical of cone contours in the other group. Hence it cannot be held, in general, that the quantitative characteristics of performance contours are diagnostic of properties intrinsic to retinal rods or cones. The slope constants ($\sigma'_{\log I}$) are the most nearly invariant indices, and despite

their apparent occurrence in two distinct groups no correlations are possible with zoological position or retinal structure, in duplex or in simplex cases.

IV

SUMMARY

Flicker contours from vertebrates (fishes to man) show that the slope parameter $\sigma'_{\log I}$ in the efficiently descriptive probability summation $100 F/F_{max.} = \int_{-\infty}^{\log I} e^{-(\log I/I_i)^2/2(\sigma')^2} \cdot d \log I$ is distributed bimodally (simple fields, "white" light), from 0.60 to 2.3, with well defined peaks at 0.80 and 1.75. This parameter is independent of $F_{max.}$, $\log I_i$, temperature, light-time fraction, and in general not greatly influenced by λ . "Rod" components of known visually duplex contours, without exception, and some "cone" contours, are in the first group; an equal number of "cone" curves are in the second group, together with one simplex "rod" contour; purely cone contours are in each group, as well as cone segments of duplex curves. No firm zoological grouping of the "cone" curves can be made, on present evidence,—although the 5 fishes used give high-slope curves, 2 amphibians low slopes, reptiles (5) either high or low, birds (2) and anthropoids (2) low-slope "cone" curves.

By subdivision of the visual image and by change of wave-length, under certain conditions, in man, and by use of the "pecten effect" in birds (and man), cone contours of the low-slope class can be transformed into curves of the high-slope group. These procedures do not fundamentally change the "rod" slopes.

Consequently, although under simple conditions they are specifically determined, the forms of the $F - \log I$ contour cannot be used as diagnostic for rod or cone functioning. It is reinforced, by new data on *Anolis* (lizard) and *Trionyx* (turtle), that an obviously duplex retina is specifically correlated with a duplex performance contour, a simplex retina with a simplex one. But no support is given to the view that the shapes of these curves are diagnostic of differences in rod or cone fundamental excitabilities, or that they describe properties of these units. In visual duplexity we have to do simply with the fact that two groups of neural effects are available; it is with their properties that we deal in measurements of duplex visual excitability.

THE MECHANISM OF ENZYME-INHIBITOR-SUBSTRATE REACTIONS*

ILLUSTRATED BY THE CHOLINESTERASE-PHYSOSTIGMINE-ACETYLCHOLINE SYSTEM

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INTRODUCTION

The phenomenon of enzyme inhibition has become a subject for pharmacological study with the growing awareness that many, if not all drugs owe their action to an ultimate combination with intracellular, extracellular or cell-surface proteins. Clark's (1) classic exposition of the manner in which drugs act upon living cells to alter their structure and function outlines the guiding principles for such investigation. More recently, the increasing interest in the mode of action of numerous bacteriostatic compounds has furthered an appreciation of the importance of enzyme inhibition as the underlying common denominator in the action of a variety of drugs. Fundamentally, this common denominator is probably not the inhibition of enzymes, as such, but rather the more general combination of a small molecule with a protein (not necessarily an enzyme), the resulting alteration of whose properties causes a change in the physiology of the cell or organism as a whole. Elucidation of the laws governing these general reactions may presumably be furthered through study of the mechanism of one type of such combination—that between enzyme and inhibitor. The pharmacologist must study such a reaction not alone from the standpoint of its inherent mechanism, but also with the realization that if he has abstracted the system from the animal to the test-tube, he must replace it again in its normal physiological environment. In other words, he must seek solutions not alone for the problems which arise under the artificial conditions of experimentation he has created, but also for the corresponding and more significant problems arising from action of the system *in vivo*. It is also true that results *in vitro* rarely will apply *in vivo* unless the artificial conditions set up in the experiment are unravelled, and appropriate changes made to fit the data obtained to the physiology of the living organism.

Straus and Goldstein (2) attempted to apply such a treatment to enzyme-

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inhibitor systems. On the basis of the mass law principles governing the reversible combination of enzymes and inhibitors, they were able to show some of the errors entailed in applying *in vitro* data to *in vivo* situations. They demonstrated that these systems could operate in three "zones of behavior", and that two of these are commonly neglected in treatments of enzyme kinetics even though they may be significant within the living cell or organism. They also showed that the common practice of diluting an enzyme-inhibitor system for determination is a crucial operation affecting the observed inhibition of the enzyme. Their theory yielded several other interesting conclusions which cannot be restated here. As the present studies are to some degree an extension and elaboration of the above mentioned work, the reader is referred to the original article for a comprehensive background of the material which is to follow.

The work of these authors, as they pointed out, was incomplete in several respects, two of which stand out prominently. *First*, it dealt only with *non-competitive* inhibition—that is, inhibition which is unaffected by the presence of substrate. Such a treatment could be applied *fully* to truly non-competitive inhibitors (although the system they chose for illustration was actually competitive), and also to enzyme-substrate combinations in which no third reactant is present; and *practically* to the case where competition, although present, is not significant. However, their treatment is not applicable to systems where substrate materially alters the enzyme-inhibitor equilibrium. *Second*, it confined itself to the study of equilibrium conditions and did not consider the attainment of equilibrium, which is a kinetic process. Time can be a very important factor and the formal application of their results based on equilibrium to a dynamic, transitional situation might be fraught with error.

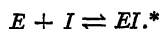
It will be the primary purpose of this paper to consider the two major problems omitted from the previous work, and further to develop the usefulness of the zone analysis of enzyme behavior. Emphasis will be placed upon the theoretical and general rather than upon the experimental and specific, but experimental data will be introduced frequently for purposes of illustration. Such data will refer to the cholinesterase-physostigmine-acetylcholine system which was also used by Straus and Goldstein; we shall therefore be able to demonstrate for a particular system the practical effects of incompleteness in their theoretical postulates. We wish to emphasize that nothing in our analysis limits its validity to any single system and it is to be hoped that the analytical methods elaborated here will be applied to others of similar type.

Zone Behavior in Competitive and Non-Competitive Systems

Non-Competitive Equilibrium.—

Let us consider a reversible enzyme-inhibitor combination in which sub-

strate plays no part—the non-competitive case represented by the reaction:



The activity of the enzyme at any time depends upon its ability to combine with a substrate. The degree of inhibition will be equal to the fraction of total enzyme centers prevented from combining with substrate, and conversely the degree of activity will be the fraction remaining free. We may designate this *fractional activity* by a , and define $a \equiv E_f/E$, where E_f and E are free and total enzyme respectively. At equilibrium we may write the mass law expression:

$$\frac{(E_f)(I_f)}{(EI)} = K_I$$

or

$$\frac{(E_f)(I - EI)}{(EI)} = K_I \quad (1)$$

* Table of Symbols Used

Symbol	Definition	Refer to page:
E, E_f	Molar concentration of <i>total</i> and <i>free</i> enzyme centers.....	531
I, I_f	Molar concentration of <i>total</i> and <i>free</i> inhibitor.....	532
S, S_f	Molar concentration of <i>total</i> and <i>free</i> substrate.....	532-33
EI	Molar concentration of enzyme-inhibitor complex.....	532
ES	Molar concentration of enzyme-substrate complex....	532
K_I, K_S	Dissociation constants of enzyme-inhibitor and enzyme-substrate complex.....	531-32
I', S'	Specific concentrations of inhibitor and substrate, defined as I/K_I and S/K_S respectively.....	532-33
E'_I, E'_S	Specific concentration of enzyme, in terms of inhibitor and of substrate, defined as E/K_I and E/K_S respectively.....	532-33
a	Fractional activity of enzyme, defined as E_f/E in non-competitive and ES/E in competitive system. See also discussion on p. 539.....	531
i	Fractional inhibition of enzyme, defined as EI/E	539
v	Observed velocity of destruction of a substrate.....	533
$V_{\max.}$	Maximal velocity of substrate destruction for a given enzyme concentration.....	533
n	Molecules of substrate or inhibitor combining with each enzyme center....	549
k_D	Velocity constant for destruction of a substrate.....	533
k_1, k_2	Forward and reverse velocity constants for the enzyme-inhibitor reaction..	562
k_3, k_4	Forward and reverse velocity constants for the enzyme-substrate reaction..	562
N	Factor of dilution; <1 for diluting a system, >1 for concentrating.....	554
t	Time in minutes	

Zones are defined as A, B, C , with respect to inhibitor or substrate in non-competitive systems ($A_I, B_I, C_I, A_S, B_S, C_S$) and to both in competitive systems ($A_I A_S, A_I B_S$, etc.).

Special Symbols Used in the Section on Destruction:

b	Initial molar concentration of physostigmine (equivalent to I).....	573
x	Amount of physostigmine destroyed in time t	573
a	Molar concentration of second reactant (either E or hydroxyl).....	573
c	An arbitrary proportionality constant.....	574

where I_f and I represent concentrations of free and total inhibitor, and EI the concentration of enzyme-inhibitor complex. Since $E = E_f + EI$ and $E_f = aE$, it follows that $EI = E(1 - a)$. Substituting this value we find that

$$I = K_I \cdot \frac{(1 - a)}{a} + (1 - a)E \quad (2)$$

Generalizing the equation to apply to any system, we eliminate K_I and introduce the terms $I' \equiv I/K_I$ and $E'_I \equiv E/K_I$, referred to as *specific* concentration of inhibitor and enzyme respectively ((2), page 563). Equation 2 then becomes

$$I' = \frac{(1 - a)}{a} + (1 - a)E'_I \quad (3B)$$

These equations are entirely identical with equations 2 and 3 of Straus and Goldstein except that for certain reasons which will be discussed below, we have used a term a , to represent fractional activity, instead of their fractional inhibition i ; here, $a = (1 - i)$.

It will be recalled that zones of behavior are established on the basis of equation 3B. It states that the total inhibitor I' is composed of two separate parts: $(1 - a)E'_I$ which is equal to $(EI)_I'$, the *combined* inhibitor; and $(1 - a)/a$, which must consequently represent the *free* inhibitor. Zone A is that zone in which essentially all the inhibitor is free and

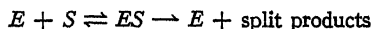
$$I' = (1 - a)/a \quad (3A)$$

In zone B the full equation must be used, while zone C is that region in which practically all the inhibitor is combined and

$$I' = (1 - a)E'_I \quad (3C)$$

The legitimacy of using 3A or 3C instead of the full equation obviously depends upon the magnitude of E'_I and boundary values of E'_I can be established, within which either simplified form may be used without exceeding any agreed upon error Δa in the dependent variable. For $\Delta a = 0.01$ the approximate boundary values of E'_I are as follows: For the boundary AB, $E'_I = 0.1$; for the boundary BC, $E'_I = 100$. (See Fig. 1 of Straus and Goldstein.)

In treating the reaction of substrate with enzyme



we must define a as equal to ES/E , since enzyme activity is directly observed by measuring the rate of destruction of substrate, which rate is proportional to the concentration of complex (ES). The term a is therefore also equal

to the ratio of observed velocity to the maximum velocity which would be attained if the enzyme were saturated with excess substrate, since,

$$\begin{aligned}v &= k_D(ES) \\V_{\max.} &= k_D E \\ \frac{v}{V_{\max.}} &= \frac{ES}{E} \equiv a\end{aligned}$$

where k_D is the velocity constant for destruction of substrate.

Proceeding exactly as in the derivation of equation 3, we arrive at an equation which is almost identical with 3B:

$$S' = \frac{a}{1-a} + aE_s' \quad (4B)$$

where, of course, $S/K_s \equiv S'$ and $E/K_s \equiv E_s'$. This equation follows exactly the same zone principles already outlined and is in fact the simplest type of reaction to analyze in terms of zone behavior; for it is entirely non-competitive and also subject to none of the errors to be discussed in connection with more complex systems. It is to be noted here that in the above derivations and throughout this paper we have considered *concentrations* of enzyme, inhibitor, and substrate as equivalent to their *activities*. Actually the mass law equations call for the use of the latter; and furthermore it is quite likely that for charged molecules (enzymes and many inhibitors and substrates) activity coefficients will vary with concentration. However information on this subject is still too incomplete to allow its inclusion in the present mathematical treatment.

What can we say regarding the real likelihood that enzyme-substrate systems will operate in one zone or another; *i.e.*, that they will be described well by either simplification or by the full equation? Since $E_s' \equiv E/K_s$, it follows that if K_s is not exceptionally small, and since the molar concentration of the protein enzyme cannot be very great (at least *in vitro*), E_s' is very likely to be less than 0.1. In that case the system will operate in zone A. That this is generally true is indicated by the fact that the classical treatment of enzyme-substrate systems (Michaelis and Menten (3)) has satisfactorily employed the zone A equation,

$$S' = a/(1-a) \quad (4A)$$

It must be borne in mind, however, that the above conditions for zone A behavior need not apply under all conditions nor for all substrates and enzymes.

In Fig. 1 we present a typical "dose-effect curve" in which the activity of an enzyme is plotted against the \log_{10} of the substrate concentration. We do not believe that the manner of portraying such experimental data is a question

to be decided by individual preference, since if clarity and ease of analysis are furthered by a particular type of plot, it should obviously be generally adopted. Yet it is interesting to find that the plot of Fig. 1, although familiar and advantageous in many respects, has not been universally accepted for describing a "dose-effect" relationship. Its advantages are: (1) it corresponds with ex-

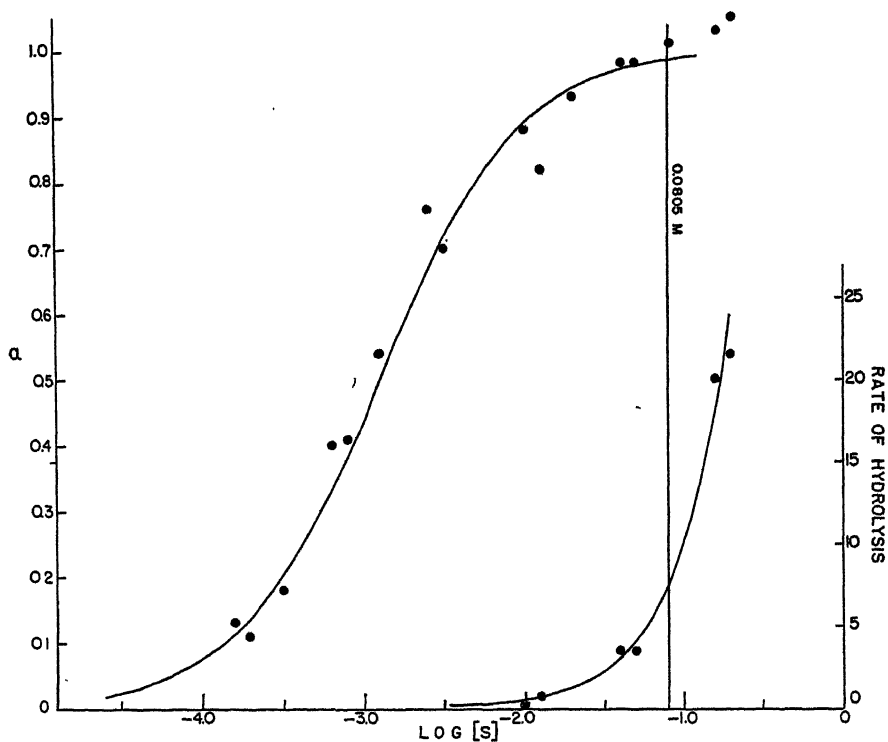


FIG. 1. Acetylcholine activity curve. Ordinate (left): fractional activity, α . Ordinate (right): non-enzymatic hydrolysis rate (cubic millimeters CO_2 per 20 minutes). Abscissa: \log_{10} of the molar acetylcholine concentration. The solid vertical line shows the standard substrate concentration used in the other experiments described.

● = averages of two or more experimental determinations.

perimental conditions in that the abscissa and ordinate respectively represent the actual independent and observed dependent variable; (2) it shows clearly the *asymptotic* nature of the typical "dose-effect" mechanism; (3) it depicts the entire range from 0 to 1 in the fractional activity of an enzyme without distortion; (4) it lends itself to a mathematical analysis in which its shape, slope, and position assume real significance—this point will be amplified in a later section.

The data for Fig. 1 are obtained from experiments in which the concentration of acetylcholine was varied and the resulting changes in velocity of acetylcholine destruction observed. (See page 560 for experimental method.) If the usually employed zone *A* equations are valid in this case, it is a comparatively simple matter to solve for V_{\max} and K_s from the relation

$$\frac{1}{v} = \frac{K_s}{V_{\max}} \cdot \frac{1}{S} + \frac{1}{V_{\max}} \quad (5)$$

modified from Michaelis and Menten, who considered only the zone *A* case (*E* does not enter the equation). Substitution into this equation gives $K_s = 1.25 \times 10^{-3}$, an acceptable value if the system really lies in zone *A*. That it does is indisputable in the present instance, for we know from other experiments (pages 543 and 544) that *E* is of the order of 10^{-8} molar or less. For a system to operate in zone *B* (*E'* greater than 0.1) when K_s is as large as 10^{-3} would require a molar enzyme concentration of at least 10^{-4} . Accepting the results obtained from equation 5 we may then express activities as $v/V_{\max} = a$ and plot the experimental points accordingly. We may also draw the theoretical zone *A* curve with $K_s = 1.25 \times 10^{-3}$ (the solid line of Fig. 1), to which the experimental points are seen to fit satisfactorily. This value of K_s agrees well with Glick's (4) 1.1×10^{-3} and Eadie's (5) 1.7×10^{-3} , and will be used throughout this paper.

The method just described for fitting a curve to the experimental points may seem excessively involved and argumentative when strictly mathematical means might better be employed. However, we wish to emphasize the contrary view that here careful reasoning is superior to the formal use of statistical methods. The generally accepted method for curve-fitting to the zone *B* equation is that proposed by Easson and Stedman (6) and used since by a number of authors including Straus and Goldstein. Their procedure is to divide the full equation by *a* or $(1 - a)$ so that

$$\frac{S'}{a} = K_s \cdot \frac{1}{1 - a} + E$$

and

$$\frac{I}{1 - a} = K_I \cdot \frac{1}{a} + E$$

One can then plot S/a against $1/(1 - a)$ or $I/(1 - a)$ against $1 - a$ and obtain a linear curve with slope equal to K and intercept equal to *E*. From these constants one can construct the theoretical curve with the desired abscissa ($\log I$) and ordinate (*a*).

But unfortunately the transformation of variables alters the equation in such a way that the curve plotted from the resulting constants may not be the

best fit to the experimental data. This is because the best fit of a linear curve to the transformed equation is *not* the same as the best fit of the original hyperbolic function to the experimental points. This is emphasized by the vastly disproportionate weights of the points at one end of the curve (when a is small in the equation for I ; when a is large in the equation for S). When one fits such a linear curve to the transformed data by means of a method of least squares (7), one is impressed by the fact that all the experimental points but a few at the crucial end of the curve could be omitted entirely with little effect on the end-result. For example, in the data for Fig. 1, a very small error in the points near $a = 1$ could so affect the constants obtained by this method that E would appear large enough to place the system in zone B . Although such a conclusion would be derived from an apparently legitimate mathematical method, it would be, as we have shown, nonetheless untenable; and the directly plotted zone A curve of Fig. 1 would be a far more truthful portrayal of the experimental data.

On account of the inherent faults in this statistical method, it is preferably not used at all, or employed with caution so as to avoid if possible the distortions which can so easily occur. Since the absolute experimental error is the same for all values of a , a curve-fitting method is required which would weight all points equally so that the standard deviation of the experimental points (*not of the transformed points*) would be minimal from the desired curve.

Although we have introduced a term $V_{\max.}$, it is important to realize that "maximal velocity" is unobtainable in this type of reaction, just as "complete inhibition" is a misnomer when an inhibitor is the independent variable. These reactions, as we have pointed out, are all asymptotic to some hypothetical value which is never actually (although it may be practically) attained. Consequently one cannot conceivably use an "excess" of substrate to "saturate" an enzyme, except in a very figurative sense. This seems to be repeating the obvious, but it may not be generally realized that significant errors can arise from the notion that a particular substrate concentration is adequate to produce a "maximal" rate of enzyme activity.

Fortunately one can predict from the zone A equation what the value of a must be for a given concentration of S , once K_s is known for any substrate. We have, for example, used 0.0805 M acetylcholine in all our studies requiring constant S and variable I ; this is equivalent to $S' \equiv S/K_s = 64.4$. Reference to equation 4A or Fig. 1 will show that this concentration ($\log S' = 1.81$) gives $a = 0.985$; that is to say, 98.5 per cent of the enzyme is saturated. Although this is quite satisfactory from a practical standpoint, casual perusal of the literature reveals substrate concentrations in general use which must result in considerably less complete enzyme saturation, no longer even approaching "maximal." The errors entailed in such experiments will be taken up in the

appropriate section dealing with suboptimal substrate concentration (page 545). We suggest that in the case of a new substrate, several quick determinations with varying concentrations of S can yield a rough figure for K_s (that concentration of S giving half the apparent maximal velocity). Then equation 4.4 will give the necessary S to achieve any desired saturation of the enzyme.

The curve at the lower right of Fig. 1 shows the non-enzymatic hydrolysis of acetylcholine as a function of acetylcholine concentration. It is a convenient coincidence that the substrate concentration we have chosen to use is just below that producing a sharp rise in the hydrolysis curve, so that although we always correct for hydrolysis, this correction remains a small one.

Competitive Equilibrium.—

By *competitive* equilibrium we mean one into which enter not only enzyme and inhibitor or enzyme and substrate, but all three elements simultaneously. Thus two separate equilibrium equations must be satisfied.

$$\frac{(E_f)(I_f)}{(EI)} = K_I \quad \text{and} \quad \frac{(E_f)(S_f)}{(ES)} = K_s$$

As before, let $(ES)/E \equiv a$ so that $(ES) = aE$. Then since $E = ES + EI + E_f$,

$$\frac{EI + E_f}{E} = (1 - a) \quad \text{and} \quad EI = (1 - a)E - E_f.$$

For (E_f) we may substitute its value $\frac{K_s(ES)}{S_f} = \frac{K_s aE}{S - aE}$.

For (I_f) substitute $(I - EI)$, and for (S_f) , $(S - ES)$.

And introducing $I' \equiv I/K_I$, $S' \equiv S/K_s$, $E_I' \equiv E/K_I$ and $E_S' \equiv E/K_s$, we have:

$$I' = \left[(S' - aE_S') \left(\frac{1 - a}{a} \right) - 1 \right] + \left[1 - a \left(1 + \frac{1}{S' - aE_S'} \right) \right] E_I' \quad (6B_I B_S)$$

Total

Free

Combined

It can be verified that the last expression is equal to $(EI)'$, the specific concentration of *combined* inhibitor. Reasoning as previously, we conclude that the first term on the right must be equal to *free* inhibitor. This is the equation describing competitive equilibrium when the system is in zone B with respect to both inhibitor and substrate. It is the most rigid form possible, since no approximations or simplifications were employed in its derivation.

The most direct simplifications to consider are those based on the equation already presented. If practically all the inhibitor is free, we may write:

$$I' = (S' - aE_S') \left(\frac{1 - a}{a} \right) - 1 \quad (6A_I B_S)$$

And likewise, if practically all is combined:

$$I' = \left[1 - a \left(1 + \frac{1}{S' - aEs'} \right) \right] E_I' \quad (6C_I B_S)$$

The more commonly used simplifications will concern S rather than I for in general it will be common to find the enzyme operating in zone A with respect to substrate ($E/K_S < 0.1$) but in another zone with respect to inhibitor (since K_S will often be much greater than K_I). In zone A_S , practically all the substrate is free, so that $S' - aEs' \doteq S'$. This allows us to write:

$$I' = \left[S' \cdot \frac{1-a}{a} - 1 \right] + \left[1 - a \left(1 + \frac{1}{S'} \right) \right] E_I' \quad (6B_I A_S)$$

and

$$I' = S' \cdot \frac{1-a}{a} - 1 \quad (6A_I A_S)$$

$$I' = \left[1 - a \left(1 + \frac{1}{S'} \right) \right] E_I' \quad (6C_I A_S)$$

We must emphasize that the validity of these simplifications with respect to substrate depends *not* upon having an "excess" of S , in terms of concentration, but rather upon K_S and E being large enough or small enough so that $Es' < 0.1$, or > 100 , regardless of the actual concentration of S employed.

The case where practically all S is combined (zone C_S) would be represented by the equation

$$S' = aEs' \quad 4C$$

If this were true, a would be equal to the ratio S'/Es' (or S/E , which is the same thing) regardless of the presence or absence of I . We would then have a valid non-competitive equation for zone C in an enzyme-substrate system, but no equation including inhibitor could be written.

Reinspection of equation 6 $C_I B_S$, which described zone C with respect to inhibitor, reveals a similar situation. If S' is very small, then a is limited to infinitesimal values and there is really no competitive inhibition, the equation itself reducing to the non-competitive form 3C. If, on the other hand, S' is large, competition is present but we are no longer in zone C , for a significant amount of I is displaced from combination to become *free* I , and the full equation (6 $B_I B_S$) must be used. It follows from this and the preceding paragraph that *competitive inhibition cannot exist in zone C, and, conversely, that if inhibition is competitive the zone must be A or B.*

A simplification of a different type is possible, if we agree to neglect (E_f) which must represent but a small fraction of the total enzyme when (EI) and

(ES) are both present and account for the major part of E . In the derivation we let $(ES)/E \equiv a$, as before, but now we let $EI/E \equiv (1 - a)$, so that (EI) is equal to $(1 - a)E$, instead of to $(1 - a)E - (E_I)$. Then by the same steps as before:

$$I' = \underset{\text{Total}}{(S' - aE_S')} \underset{\text{Free}}{\left(\frac{1-a}{a}\right)} + \underset{\text{Combined}}{(1-a)E_I'} \quad (7B_1B_S)$$

The meaningful zone forms are then:

$$I' = (S' - aE_S') \left(\frac{1-a}{a}\right) \quad (7A_1B_S)$$

$$I' = S' \cdot \frac{1-a}{a} + (1-a)E_I' \quad (7B_1A_S)$$

$$I' = S' \cdot \frac{1-a}{a} \quad (7A_1A_S)$$

The zone C form reduces, as we should expect, to the ordinary non-competitive equation 3C.

This is a convenient time to return to a discussion of the term a which we have been using instead of the older i , introduced by Easson and Stedman, and used also by Straus and Goldstein. The reason for the change is quite basic and should be clear now that the various equations have been derived. In studying the activity of an enzyme, we can only observe its activity as reflected in its ability to act upon a substrate. The observed velocity is proportional to the concentration of enzyme-substrate complex [$v = k_D (ES)$] so that a is both an experimental observation (v/V_{\max}) and a description of the ratio of enzyme-substrate complex to total enzyme (ES/E).¹ The term i , on the other hand, describes the fraction of enzyme-inhibitor complex ($i \equiv EI/E$), of which we have no direct measure, so that we are forced to measure a experimentally and then substitute $i = (1 - a)$. We have just seen, moreover, that this is itself only an approximation which ignores free enzyme entirely. For the non-competitive case it was adequate, because the enzyme consisted of only two parts — EI , which was inactive; and E_I , which could be considered "active" by virtue of its ability to combine with a "saturating concentration" of substrate. We now see that in the competitive equilibrium a part of the enzyme may combine with neither inhibitor nor substrate. In fact, despite a negligible concentration of inhibitor the activity may nevertheless be very low

¹ For convenience in describing non-competitive enzyme-inhibitor systems we had to allow a to equal E_I/E (since no ES is present), but if this altered definition is remembered, no confusion should result.

because so little substrate is present. Therefore, since we are interested in enzyme *activity* and not in inhibitor-caused *inactivity*, we employ the term a , and suggest its general adoption.

Although a slight error is introduced (see below) we shall employ the simple forms of the equations in which (E_I') has been neglected. The equation which will find most general use is equation 7 $B_I A_S$ for reasons which have already been pointed out. This equation is identical in form with equation 3B except for the multiplier S' . The zone boundaries for equation 3B were derived by Straus and Goldstein and the steps need not be repeated here. In the non-competitive case the zone A form could be used when $E_I' < 0.1$. In the present case, then, the zone A form is valid when $E_I'/S' < 0.1$:

$$I' = S' \cdot \frac{1-a}{a} \quad \text{when } E_I'/S' < 0.1$$

$$I' = (1-a)E_I' \quad \text{when } E_I'/S' > 100.$$

Furthermore the "dose-effect" curves are identical for the competitive and non-competitive cases except for a shift on the $\log I'$ axis.

Fig. 2 is a plot of $\log I'$ as abscissa against a as ordinate for various values of E_I' , showing the characteristic shapes and positions of the "dose-effect" curves in each zone. For the non-competitive case one ignores the term S' entirely so that the figure is then identical with Fig. 2 of Straus and Goldstein. It will be recalled that all values of $E_I' < 0.1$ are represented by a single symmetrical limiting zone A curve, inflecting at $a = 0.5$, and with slope 0.575 at that point. The curves for zone B become steeper and spaced out until in zone C, when $E_I' > 100$, they are parallel and essentially simple logarithmic curves (since $I' = (1-a)E_I'$). To use these same curves for competitive inhibition in any system, one simply multiplies the value of E_I' by S' and I' by S' (adding $\log S'$ to $\log I'$) as indicated. This shifts the curves in a horizontal direction but does not alter them otherwise. All details in the interpretation of these curves will be essentially as presented by Straus and Goldstein for the non-competitive case.

The curve of competitive equilibrium in the system cholinesterase-physostigmine-acetylcholine is represented by the experimental points plotted about the curve C in Fig. 3, the data for which are found in Table II*. The effect of introducing the usual large concentrations of S is always in the direction of *reducing the zone* in which the non-competitive system might have operated. Let us assume $E_I' > 0.1$ so that the non-competitive system is in zone B; E_I'/S' will probably still be less than 0.1 (because of the magnitude of S' —64.4

* See Appendix for Tables II to VI.

in our experiments), so that the competitive system is in zone *A*. We have already discussed the effect of *S* in reducing a zone *C* system to zone *B*.

With regard to curve *C* of Fig. 3, it was readily apparent from the concentration of *S* and the approximate value of K_I , that, although the system might have been in zone *B* under non-competitive conditions, it *must* now be in zone

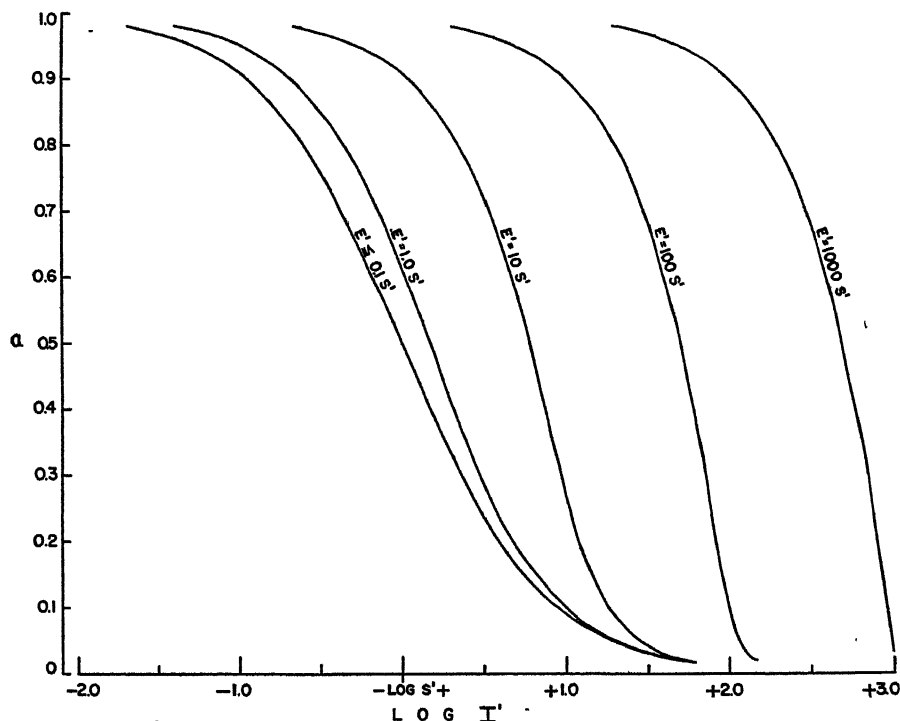


FIG. 2. Zone curves for $n = 1$. Fractional activity, a , as a function of $\log_{10} I'$ for discrete values of E_I' . These curves represent both non-competitive and competitive equilibrium. For competitive equilibrium the figures on the abscissa are added to \log_{10} of the specific concentration of substrate (S') as shown. For non-competitive inhibition substitute 0 for $\log S'$ on the abscissa, and 1 for S' on the curves themselves, which will then describe the equilibrium in the absence of substrate.

A, unless E exceeded 10^{-4} molar. We therefore drew the best zone *A* curve for the experimental points, and the fit is seen to be quite satisfactory. Knowing S and K_S (from the experiment of Fig. 1) and reading off I' when $a = 0.5$, we can then calculate the value of K_I :

$$\frac{I}{K_I} = \frac{S}{K_S} \cdot \frac{1-a}{a}$$

and when $\alpha = 0.5$,

$$K_I = \frac{K_S I}{S}.$$

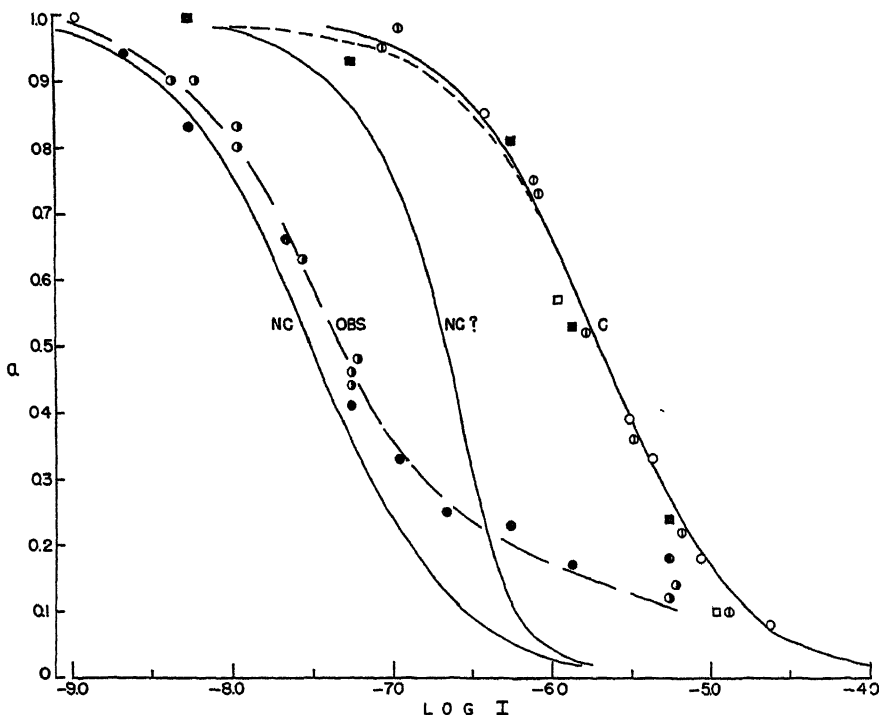


FIG. 3. Competitive equilibrium (experimental and theoretical). For all curves: a is plotted as a function of \log_{10} of the molar physostigmine concentration in the reaction mixture.

OBS: the curve drawn through observed activities as determined 3 to 23 minutes after substrate addition. \bullet = duplicate determination; \circ = single determination.

C: the zone A curve fitted to experimental points representing 20 minute readings at full equilibrium of the E - I - S system. \odot = duplicate determination; \circ = single determination. \square and \blacksquare = activities approached by the curves of Figs. 8 and 9 respectively at equilibrium. The broken line of the upper part of curve C indicates the slight falling off observed at this substrate concentration when E_I is not neglected in deriving the equation.

NC: the theoretical zone A non-competitive curve for the value of K_I obtained from curve C. This curve correctly lies to the left of the 3 to 23 minute curve OBS.

NC?: the absurd non-competitive curve for the value of E and K_I obtained through application of the statistical methods described in the text, to curve C. The absurdity of this curve consists in its position far to the right of OBS.

K_I for dog serum, obtained in this way, is found to be 3.11×10^{-8} and we believe that the excellent fit of the experimental points to the curve, the independence of K_I from E in this zone, and the conditions of complete competitive equilibrium make this constant subject to little error.

As Straus and Goldstein stated, and as is obvious from equation 7 $A_I A_S$ although K can be obtained in zone A , there is no way of arriving at an estimate of E , which does not appear in the equation. The points on the curve OBS in Fig. 3 represent the data obtained from readings in the period 3 to 23 minutes after addition of substrate. Most investigators who have sought a value of E have used this type of curve and assumed that it was essentially non-competitive. Applying the non-competitive equations, none have had difficulty in arriving at a value for E . Straus and Goldstein, on the basis of just such methods, found $E = 2.7 \times 10^{-8}$ in 22.2 per cent horse serum, giving $E_I' = 0.73$, so that the system apparently acted in the lower part of zone B . It is now clear from the experiments presently to be described that the 3 to 23 minute curve is certainly not non-competitive, but represents a transitional stage in the entrance of substrate into the equilibrium. In other words, in Fig. 3, the *true* non-competitive curve relating a to $\log I'$ in the absence of substrate, lies somewhere to the left of the curve OBS, while the final competitive equilibrium is represented by curve C, far to the right. To make this clearer, let us assume a certain concentration of inhibitor ($\log I = -7$) equilibrated with enzyme. When substrate is added for the 3 to 23 minute determination, a is found to be 0.35. This means that a must have been *less* than 0.35 at the moment of substrate addition. When the new competitive inhibition has been attained, with this same concentration of inhibitor the activity has risen to 0.94. This "competition effect" will be analyzed at great length subsequently; it is brought in here only to show that the common 3 to 23 minute curve is unreliable for the determination of any constant, least of all E .

Although not as satisfactory as a definitive solution would be, there is still one approach we can make to the problem of determining E . It is clear from the above discussion that every point on the true non-competitive curve (NC) must lie to the left of the curve OBS. Let us take the point $a = 0.5$ on OBS; the corresponding value of I is 4.37×10^{-8} . We may then say that:

$$I_{NC} < 4.37 \times 10^{-8} \quad \text{when } a = 0.5.$$

Then

$$< 4.37 \times 10^{-8} = 3.11 \times 10^{-8} + 0.5E \quad (\text{Equation 2B})$$

and

$$E < 2.52 \times 10^{-8}.$$

If we apply the same reasoning to the point $a = 0.7$, we can show that $E < 1.8 \times 10^{-8}$. By taking larger values of a , we might delimit E still further, but the scatter of the experimental points becomes too great in this range to make such a procedure reliable.

It is of some interest to note that had we applied the illegitimate statistical method discussed on page 535 to the data of curve C, we would have arrived at a definite and not negligible value of E (3.7×10^{-8}), large enough to place the system in zone B. One might easily be misled by such a result were the process of reasoning omitted, whereby it was shown that the system *must* be in zone A. Fortunately we are able further to show the impossibility of so large an E by plotting the non-competitive curve for that value. This is the curve NC? in Fig. 3 which is absurd by virtue of its position considerably to the *right* of the experimental curve of 3 to 23 minute readings (OBS).

In summary we may say that while K_I has been determined definitely, E has not. Its maximum value may be 1.8×10^{-8} (in 4.54 per cent dog serum) in which case $E_I' = 0.58$ and the non-competitive system is in the lower part of zone B; or E_I' may be less than 0.1 ($E < 3.1 \times 10^{-9}$) in which case the system is in zone A and E cannot possibly be determined from this type of experiment. The curve NC in Fig. 3 represents the non-competitive curve if $E_I' < 0.1$; the curve for $E_I' = 0.58$ would lie between NC and OBS. The competitive system is in zone A in any case, so that E cannot be determined from competitive data.

It is of interest to examine the equation $7 A_I A_S$, which represents a common situation, and precisely the one depicted in our curve C of Fig. 3. The equation may be re-written:

$$\frac{I'}{S'} = \frac{1-a}{a}$$

This states, as has already been pointed out, that a is a function of the ratio I'/S' , which is in turn equal to the product $\frac{I}{S} \cdot \frac{K_S}{K_I}$. When $a = 0.5$, $\frac{I}{S} = \frac{K_I}{K_S}$, in other words to obtain half inhibition of the enzyme requires no absolute amount of either substrate or inhibitor, but a fixed ratio of one to the other. Each molecule of I "neutralizes" a number of S molecules equal to the ratio of their respective dissociation constants.

From a somewhat different standpoint we may rewrite the equation:

$$\frac{1-a}{I'} = \frac{a}{S'}$$

$$\frac{\frac{EI}{E}}{I'} = \frac{\frac{ES}{E}}{S'}$$

and

$$\frac{\frac{EI}{I}}{\frac{ES}{S}} = \frac{K_S}{K_I}$$

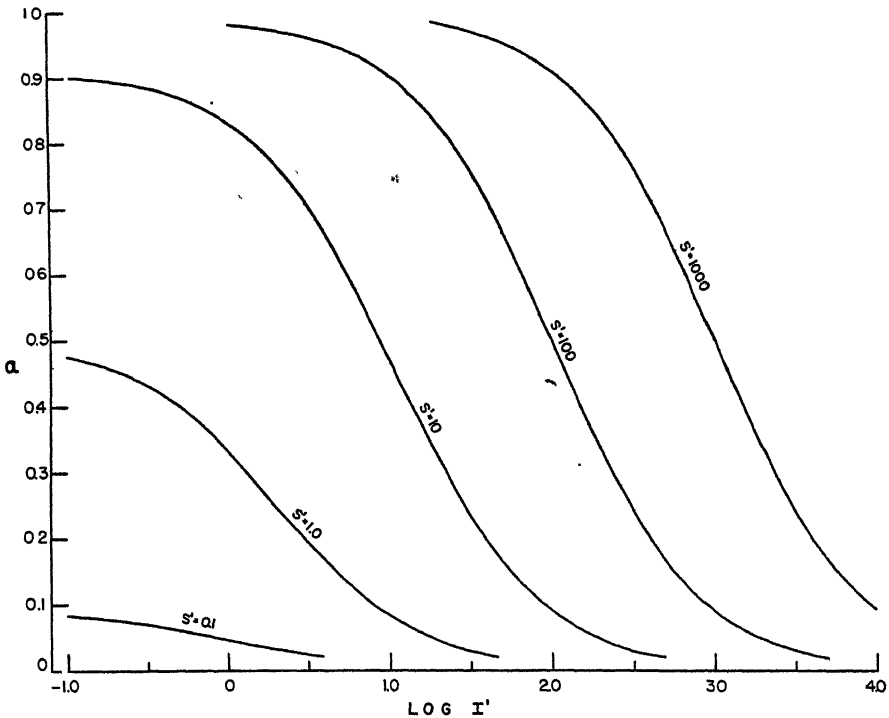


FIG. 4. Suboptimal substrate concentration. Fractional activity, a , as a function of $\log_{10} I'$ for discrete values of S' .

This states that the ratio of the combined fractions of inhibitor and enzyme respectively is equal (for all values of a) to the inverse ratio of their dissociation constants.

Suboptimal Substrate Concentration.—

Our previous discussion touched upon a few of the effects of suboptimal substrate concentration. These effects are best illustrated by Fig. 4, in which a is plotted against $\log I'$ for various discrete values of S' . To keep the discussion reasonably simple, we have assumed zone A_1A_2 . We may not, however, simplify by neglecting E_f , for as we have seen it is precisely when S' is small that the enzyme may be unsaturated (and E_f significant) even though both S and I be present. In Fig. 4 we find that while the curves for high S' resemble

the zone *A* competitive curve of Fig. 2, as S' falls off, the curves become asymptotic to some lower value than $a = 1.0$. Actually, as I' approaches zero, each curve approaches that activity which would be predicted by Fig. 1 and equation 4*A* for that particular specific concentration of substrate. This is seen in the approach of equation 6*A*_{*I*}*A*_{*S*}

$$I' = S' \cdot \frac{1 - a}{a} - 1$$

to

$$S' = \frac{a}{1 - a} \quad \text{as } I' \rightarrow 0.$$

Thus, the activity a , in a competitive system does not vary from 0 to 1, but only from 0 to $S'/(1 + S')$ (which approaches 1 as $S' \rightarrow \infty$). The characteristic skewed shape of the curve is observable even at the high S' we have used; this can be observed in the falling off of the upper part of curve C (broken line) in Fig. 3, to approach the value $a = 0.985$.

The competitive curves of Fig. 2 are therefore wholly valid only when S' is very large, so that E_f may be neglected. These curves have characteristic slopes, which are the same in each zone as those found by Straus and Goldstein for the non-competitive curves—varying from 0.575 in zone *A* to 1.151 in zone *C*. We have already seen that when S' is suboptimal these slopes tend to fall off, a phenomenon that is only demonstrated in the full equations which include E_f . Equation 6 *A*_{*I*}*A*_{*S*} can be differentiated to give

$$-\frac{da}{d \log I'} = \frac{\left[S' \cdot \frac{1 - a}{a} - 1 \right] a^2}{S'} \quad (8)$$

and when $a = 0.5$:

$$-\frac{da}{d \log I'} = 0.575 \frac{S' - 1}{S'}$$

Here when S' is very large the slope is 0.575, but it becomes progressively smaller as S' is reduced. Equation 8 shows that the falling off of slope when S' is made smaller applies to all parts of the curve but is most marked for the higher values of a .

It should now be evident that with suboptimal substrate concentrations, the uninhibited enzyme activity is no longer equal to $V_{\max.}$. Consequently for any enzyme one must first determine the true $V_{\max.}$ experimentally, or, knowing the constants, must calculate it on the basis of the proper equation. One must then express all observed velocities in terms of the true a . Reference to the curve $S' = 1$ in Fig. 4 shows how great would be the error of considering

as V_{\max} , that velocity attained by uninhibited enzyme at this substrate concentration; activity values expressed on this basis would err by about 100 per cent and would be worthless for analytical purposes.

The curves of Fig. 4 show certain characteristic features. In the region $I' > 100$, the curves follow equation 7 A_1A_2 ; it is the region where any given inhibition can be maintained by varying inhibitor and substrate together without changing their ratio. In the region $I' < 0.1$, all the curves have become practically horizontal; this means that activity is now determined only by substrate, changes in inhibitor concentration having insignificant effect.

These curves also show the effect of diluting a competitive system. The reader should consult pages 568-570 of Straus and Goldstein for the method of determining dilution effect with curves of this type. The results, expressed qualitatively, are as follows: There is no change in a on dilution when $I' > 100$. There is increasing change when $100 > I' > 0.1$; and the maximal decrease in activity on dilution (we refer, of course, to a decrease in a , not to the normally expected decrease in observed velocity) occurs in the region where $I' < 0.1$. The dilution effect is a result of the dissociation which occurs on diluting a reversibly associated complex. Here EI and ES both dissociate, but the observed decrease in fractional activity reflects the dissociation of ES alone.

A vertical segment of Fig. 4 at any point shows the variation in a with changing S' for a given value of I' . This relationship could more easily be demonstrated by plotting a against $\log S'$ for discrete values of I' .

Let us now consider the case of an enzyme which works upon a moderately small concentration of substrate *in vivo*; e.g., $S' = 0.1$ (for acetylcholine bromide this is equal to 28 micrograms per cc.). Reference to Fig. 4 reveals that the maximum possible activity with this substrate concentration is about 0.10. To reduce this activity by one-half requires that I' be somewhat less than 1.0. On the other hand, if with the same enzyme *in vitro* S' were 100, allowing a maximum activity of 0.99, we should need $I' = 100$ to reduce the activity by one-half. Thus inhibitor is most potent when very little substrate is present.

Now let us suppose that an enzyme determined *in vitro* against $S' = 100$ shows an activity $a = 0.5$. Fig. 4 shows that this same concentration of inhibitor has an enormously greater effect when S' is small. For example, if $S' = 1.0$, activity will be reduced to 0.01, which is 2 per cent of the maximal rate for this substrate concentration; and if $S' < 0.1$, the activity is reduced to practically nil.²

These few examples demonstrate that one can hardly expect any correlation

² The assumption is made in this whole discussion that the *in vitro* determinations are done in undiluted serum, or that appropriate dilution corrections have been applied.

between *in vitro* data with high substrate concentration and those *in vivo* where S' may be exceedingly small. But we know so little about actual substrate concentrations *in vivo* that it does not seem profitable to explore the quantitative aspects of this question any further at this time.

The fractional activity, a , of an enzyme is a direct measure of the rate of hydrolysis of the substrate. Since

$$aE = (ES)$$

and

$$-\frac{dS}{dt} = k_D(ES)$$

it follows that

$$a = \frac{1}{k_DE} \left(-\frac{dS}{dt} \right)$$

and

$$k_DE = \left(-\frac{dS}{dt} \right)_{a=1}$$

For 4.54 per cent dog serum and acetylcholine, we find that $k_DE = 0.0635$ mm per liter reaction mixture per minute. A full treatment of the destruction of substrate by enzyme will be found on page 570 where physostigmine is considered as the substrate. We merely wish to show here that a at any time is proportional to the absolute velocity of substrate hydrolysis. We have already shown that what would be an ineffective inhibitor concentration with excess substrate can produce a marked decrease in activity when substrate concentration is small. Thus the absolute velocity of hydrolysis of a substrate like acetylcholine in the serum should be so decreased that appreciable accumulation of this substance ought to occur with doses of physostigmine barely capable of causing demonstrable inhibition *in vitro*. However, we do not know how much accumulation in serum is necessary to produce physiological effects; and further, as the serum substrate concentration increases, there is a parallel increase in its enzymatic rate of destruction, thereby antagonizing the augmented accumulation rate.

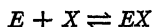
An interesting observation can be made with reference to the function of the cholinesterase, or any similar enzyme, in the serum. We know that S' in serum is likely to be very much smaller than 0.1, the value used in our previous examples. (For an acetylcholine concentration of 1:200 million, $S' < 0.0001$!) The maximum activity obtainable is consequently very much less than 0.10. The absolute rate of hydrolysis is therefore extremely small, and it might appear at first sight that practically all the enzyme present, being uncombined

with substrate, were being wasted. One must recall, however, that a represents the *relative* saturation of the enzyme; if only one-tenth of the enzyme were present, a would still have the same value (since changing E in zone A does not alter a), but the absolute velocity of hydrolysis, which was previously $ak_D E$, would now be only $ak_D(0.1E)$, or one-tenth its previous value. It is apparent, then, that to produce the necessary rate of hydrolysis of a minute concentration of substrate, *i.e.* to hold the circulating substrate concentration down to an extremely low level, one must have a tremendous "excess" of enzyme, most of which will not (at a given moment) even be combined with the substrate molecules.

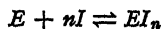
The foregoing discussion of suboptimal substrate concentration demonstrates that there is no particular reason why an investigator should work with an "excess" of substrate. It is true that the classical enzyme-substrate-inhibitor equations omitted consideration of E_f with the result that they would describe the system only when substrate was present in high concentration. Provided the descriptive equations are adequate, and the desired range of activity is covered, one may select any substrate concentration. The chief advantage of large S' is that more even coverage of the whole range of activities can be obtained. Whatever one's choice of substrate concentration, the fact remains that the results will probably not in any event be applicable to the very different concentrations obtaining under physiological conditions.

Use of Zone Phenomena for Determining Reaction Mechanism.—

All the preceding discussion has been based upon the combination of a single molecule of substrate or inhibitor with one molecule of enzyme,



Straus and Goldstein showed that it was possible to extend their analysis to systems of greater complexity, in which n molecules of a substance X combined with each molecule of enzyme (n not necessarily an integer). For the non-competitive case, if we consider



and the mass law equation:

$$\frac{(E_f)(I_f)^n}{(EI_n)} = K_I$$

we can proceed, by the same steps as were used to derive equation 3B, to the general equation:

$$I' = \sqrt[n]{\frac{1-a}{a}} + n(1-a)E_f' \quad (9)$$

where we have defined

$$I' = \frac{I}{\sqrt[n]{K_I}}, \quad \text{etc.},$$

Similarly

$$S' = \sqrt[n]{\frac{a}{1-a}} + naE_I' \quad (10)$$

For each value of n , there will be a distinct set of "dose-effect" curves, whose characteristic slopes, shapes, zone boundaries, etc., will depend directly upon n .

Slopes can be found by differentiating a with respect to $\log I'$:

$$-\frac{da}{d \log I'} = 2.303 \left[\frac{na^2}{\sqrt[n-1]{\frac{a}{1-a}} + n^2 a^2 E_I'} \right] \left[\sqrt[n]{\frac{1-a}{a}} + n(1-a)E_I' \right] \quad (11)$$

For the slope at the midpoint, set $a = 0.5$, so that:

$$-\frac{da}{d \log I'} = 2.303 \times \frac{0.25n(1 + 0.5nE_I')}{1 + 0.25n^2 E_I'}$$

The limits of variation in slope are given by:

$$\lim \left(-\frac{da}{d \log I'} \right)_{E_I' \rightarrow 0} \quad \text{and} \quad \lim \left(-\frac{da}{d \log I'} \right)_{E_I' \rightarrow \infty}$$

Thus, if $n = \frac{1}{2}$, slope varies from 0.288 to 1.151;

if $n = 1$, slope varies from 0.575 to 1.151;

if $n = 2$, slope is 1.151 in all zones.

In zone *A*, slope is equal to 0.575 n . Thus, while in zone *C* the slope is the same for every n , in zone *A* the variation is so great that slope might well be used as a criterion for the determination of n , a point to which we shall later return.

From a practical standpoint n does not vary very greatly. For enzyme-substrate and enzyme-inhibitor reactions it is likely to be either 1 or 2, or possibly $\frac{1}{2}$ (meaning that one molecule of *S* or *I* combines with two molecules of *E*). We shall consider in some detail the case $n = 2$, for the reaction $E + 2I \rightleftharpoons EI_2$, since it has been seriously proposed as the mechanism of several enzyme-inhibitor (and enzyme-substrate) combinations.

Fig. 5 shows the "dose-effect" curves when $n = 2$, for various values of E_I' , plotted according to equation 9. The general similarity between these and the curves of Fig. 2 (where $n = 1$) is evident, but we wish to stress here certain striking differences: (1) The curves appear noticeably steeper; actually it will be seen that those for the smaller values of E_I' are twice as steep as the corresponding curves of Fig. 2. (2) The curves attain a maximum spacing much

more quickly; *i.e.*, at smaller values of E_I' . (3) In the region where E_I' is great, the position of the curves on the $\log I'$ axis has shifted $\log n$ units (here 0.3) to the right.

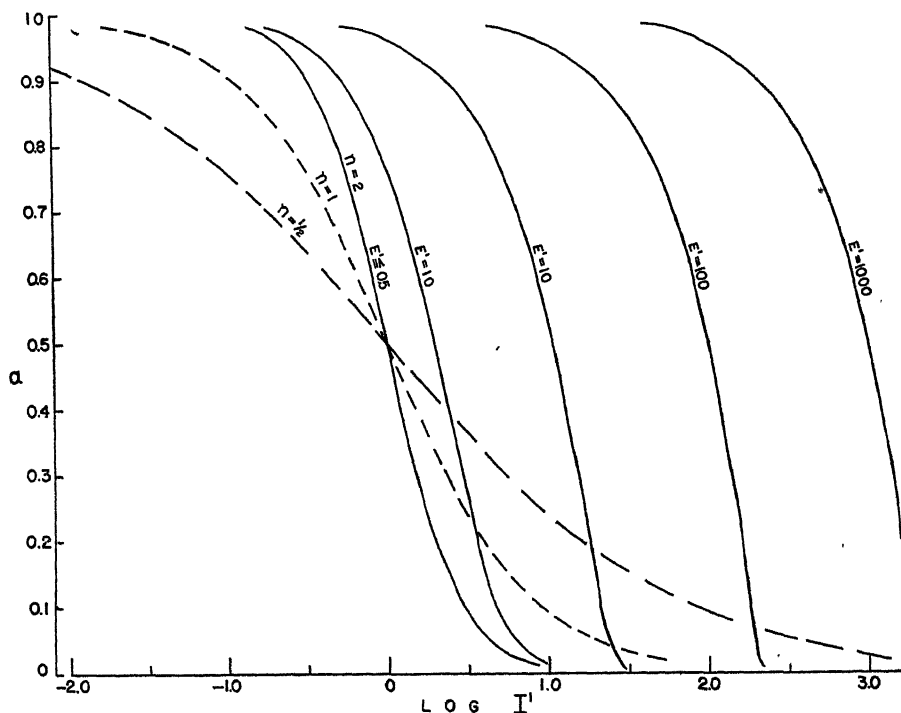


FIG. 5. Zone curves for $n = 2$. Fractional activity, a , as a function of $\log_{10} I'$ for discrete values of E_I' , when $n = 2$. The zone A curves for $n = 1$ and $n = \frac{1}{2}$ are also shown for comparison. These curves are non-competitive but S' could properly be introduced exactly as in Fig. 2.

Of these three distinctive features we have already taken up the question of *slope*. The *spacing* of the curves is determined by the zone boundaries, which are shown in Fig. 6. This figure is plotted by setting the full equation

$$I' = \sqrt{\frac{1-a}{a}} + 2(1-a)E_I'$$

equal to either simplified form *with an acceptable increment of error in a* . Thus for the boundary between zones A and B:

$$\sqrt{\frac{1-a}{a}} + 2(1-a)E_I' = \sqrt{\frac{1-(a+\Delta a)}{a+\Delta a}}$$

and for the boundary between zones *B* and *C*:

$$\sqrt{\frac{1-a}{a}} + 2(1-a)E_I' = 2(1-a-\Delta a)E_I'$$

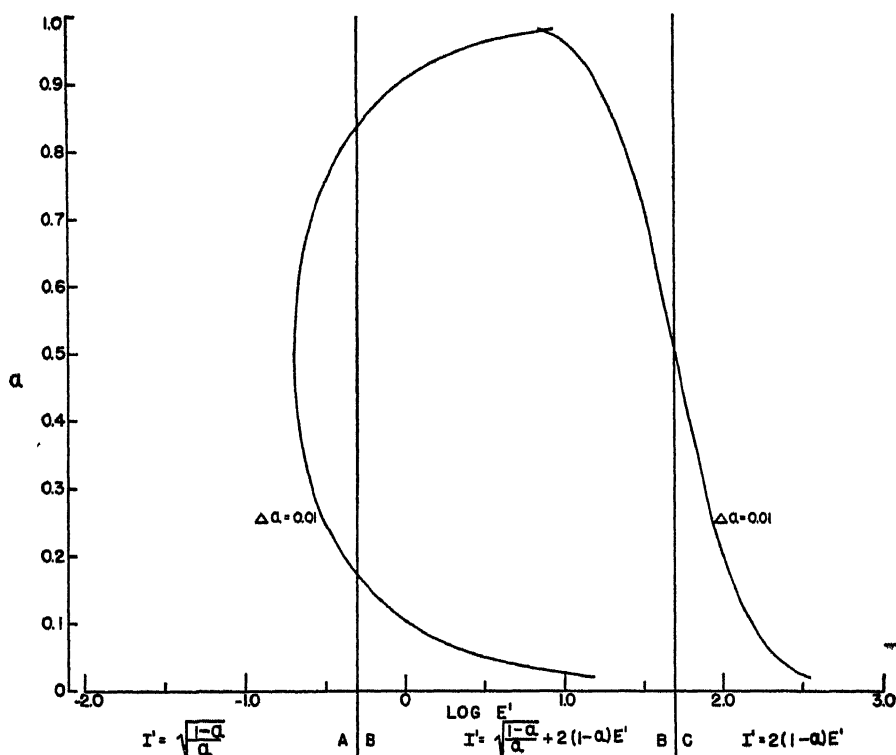


FIG. 6. Zone boundaries for $n = 2$. The two boundary curves show the exact relation between $\log E_I'$ and a for the condition that either simplification of the zone *B* equation be used without exceeding an error of 0.01 in the value of a . The vertical lines are practical zone boundaries arbitrarily adopted, neglecting the variation in a . To the left lies zone *A*; in the center zone *B*; to the right zone *C*.

The acceptable error Δa , is chosen to be 0.01. Fig. 6 shows the values of E_I' for which the simplified forms may be used so that Δa at any given a will not exceed 0.01. For practical use, we settle upon a rough average E_I' for each zone boundary: $E_I' < 0.5$ and $E_I' > 50$ for the boundaries *AB* and *BC* respectively; these boundaries should be satisfactory, provided reasonable values of a are not exceeded. Comparison with the zone boundaries in the case $n = 1$ allows one to conclude that as n is increased, zone *B* becomes narrower, while zones *A* and *C* both expand. Consequently a system which would operate

in the lowest part of zone *B* if n were equal to 1 would lie in zone *A* if n were 2; and in similar fashion a system thought to lie at the upper part of zone *B* would really be in zone *C* if n were 2.³

The shift in the position of the curves to the right, if n is increased, and to the left if n is decreased, is first noticeable in zone *B*, and becomes maximal in zone *C*, where it is equal to $\log n$. That there is no such shift in zone *A* is shown by the two zone *A* curves in Fig. 5 (broken lines) for $n = 1$ and $n = \frac{1}{2}$, whose mid-points are identical with that of the curve for $n = 2$.

What does this tell us about the relative potency of inhibitor or substrate operating according to the mechanism $n = 1$, or $n = 2$? It reveals that in zones *B* and *C*, inhibitor is less effective when n is greater, a higher concentration of *I* being required to produce half-inhibition. Since it seems likely that an enzyme such as cholinesterase (which is known to be highly concentrated at the synaptic regions) operates in zone *C* *in vivo*, we must modify Eadie's (5) conclusion that it is "possible to attain practically complete inhibition much more readily" since "the number of enzyme molecules blocked increases with the square of the inhibitor concentration, rather than with the first power." The decrease in inhibitor potency as n is increased in zone *C* is a striking example of a conclusion which may have physiological significance and which could not have been derived from classical zone *A* principles. Furthermore, even in zone *A* there is not any absolute increase in inhibitor potency when $n = 2$, and reference to Fig. 5 shows that in this zone, while a given inhibition can be obtained with less I' for activities below 0.5, the reverse is actually true when a is greater than 0.5. The only "increase in potency" consists in the fact, reflected by the doubled slope, that the entire range of inhibition can be covered by a relatively small change in I' , and it is only in this sense that Eadie's statement is correct.

The equations for competitive inhibition for any n can be derived by combining the methods employed in the derivation of equations 6 and 9, yielding:

$$I' = \sqrt[n]{(S' - naE_S')^n \cdot \frac{1-a}{a} - 1 + n \left[1 - a \left(1 + \frac{1}{(S' - naE_S')^n} \right) \right] E_I'} \quad (12)$$

All the usual simplifications and approximations can be applied to this equation and the resulting expressions will give curves bearing the same relation

³ The actual values of E_I' for different values of n are entirely comparable. This is because on the one hand we define $E_I' \equiv E/\sqrt[n]{K_I}$, while on the other, our determination of K_I is based on the concentration of *I* when $a = 0.5$, in the zone *A* form of equation 9, where $K_I = I^n$. As a result the numerical value of E_I' is the same for a given molar *E*, regardless of the value attributed to n .

to the non-competitive ones as we have already demonstrated in detail for the case $n = 1$. The slope of the competitive curve for $n = 2$ can be shown to be:

$$-\frac{da}{d \log I'} = \frac{\sqrt{S' \cdot \frac{1-a}{a}} - 1 + 2 \left[(1-a) - \frac{a}{S'} \right] E_I'}{\frac{S'}{2a^2 \sqrt{S' \cdot \frac{1-a}{a}} - 1} + 2E_I' \left(1 + \frac{1}{S'} \right)} \quad (13)$$

and when $a = 0.5$, this reduces to:

$$-\frac{da}{d \log I'} = \frac{\sqrt{S' - 1} + E_I' \left[1 - \frac{1}{S'} \right]}{\frac{S'}{0.5 \sqrt{S' - 1}} + 2E_I' \left(1 + \frac{1}{S'} \right)}$$

which yields the familiar slope of 1.151 in all zones when S' is sufficiently large. We have assumed here that the *substrate* combines with enzyme according to a 1:1 mechanism, but the result would be the same regardless of the value of n in the substrate-enzyme combination.

Straus and Goldstein pointed out that the dilution effect might be expected to change significantly if the value of n were altered, and they presented a general equation for dilution, in zone A , which can be modified to:

$$a_2 = \frac{a_1}{1 - (N^n + 1)(1 - a_1)} \quad (14)$$

where N is the factor of dilution,

a_1 is the original activity at concentration E_1

a_2 is the activity at concentration NE_1

The equation for dilution in zone B , which would correspond with their equation 7B, involves solution of a cubic which we believe is too complicated to be of any real use. Practical dilution curves for zone B can be constructed, however, from the "dose-effect" curves for various desired values of E_I' (e.g., those in Fig. 5). This has been done for the case $E_I' = 0.73$ in 22.2 per cent serum, to correspond with the dilution effect found by Straus and Goldstein, assuming n to be 1. The results at this value of E_I' are so little different from those in zone A that we need not be concerned here with these authors' probable over-estimation of E_I' , which has already been thoroughly discussed. Fig. 7 shows the effect of diluting such a system, where $n = 2$; it is read and interpreted in the same way as Straus and Goldstein's Fig. 3, with which it should be compared. The chief conclusion to be drawn from such a comparison, and one that (for zone A) could have been predicted from equation 14, is that the dissociation which occurs on dilution is more marked the greater is the value of n . *The increase of activity on diluting an enzyme-inhibitor mixture is thus considerably greater when $n = 2$ than when $n = 1$.*

We have gone into considerable detail in analyzing the general effect of variation in n , and the specific comparison of the case $n = 2$ with $n = 1$ to provide a firm basis for the following discussion of the use of zone phenomena in determining the mechanism of an enzyme-inhibitor or enzyme-substrate combination.

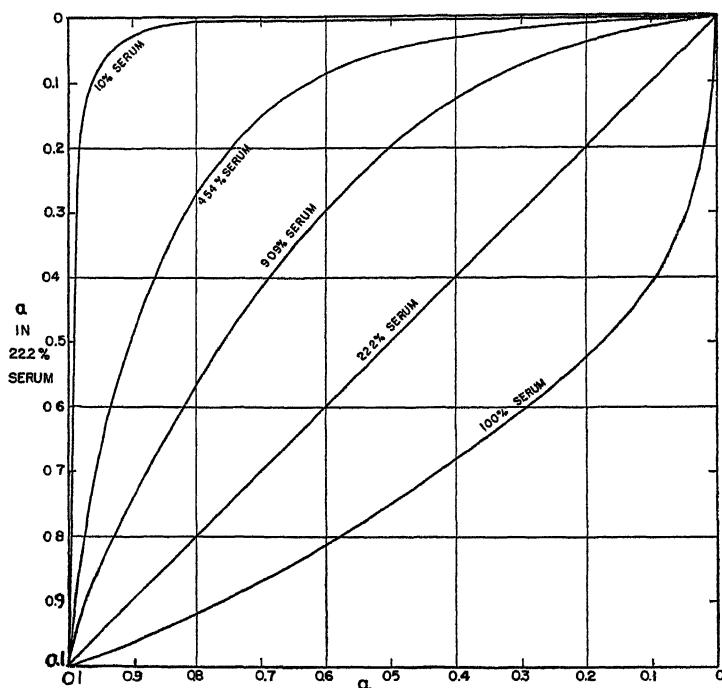


FIG. 7. Dilution effect for $n = 2$. Ordinate: fractional activity in 22.2 per cent serum. Abscissa: fractional activity at any serum concentration. Dilution or concentration of an enzyme-inhibitor mixture is represented by travelling horizontally from the original to the final serum concentration and reading off the initial and final activities from the abscissa. The ordinate was used in constructing the figure and is convenient if one is interested in activity in 22.2 per cent serum, but it is not essential in using the figure. This figure is constructed for $E_I' = 0.73$ in 22.2 per cent serum, to correspond with Fig. 3 of Straus and Goldstein.

There appears to be a widespread opinion to the effect that since (1) pharmacological experimentation usually yields valid data only in the middle portion of a range of observed effects and not at the extremes, and since (2) many different curves representing entirely different functions relating the two variables may be roughly alike in their middle portions, and since (3) the precision of the pharmacologist's observations is not comparable to that obtained by the physical chemist, one should hesitate to draw conclusions about the mechanism of a

reaction from observed data. We can see little merit in this reasoning for, while the first point may be generally true, the second need not be, while the third may not even constitute a handicap provided one knows the error to which one's data are subject.

The most direct criterion of the value of n is the slope of the "dose-effect" plot, and this slope is the same regardless of the units used to express inhibitor concentration. Here the differences in slope are so great that confusion is hardly possible unless the experimental points are unusually scattered. That the points through which the curve of Fig. 1 is drawn could not conceivably lie on a curve with double or half the slope is hardly open to doubt. Thus the actual slope, 0.575, allows one confidently to assign the value $n = 1$ to the enzyme-substrate combination in this case. We have shown that the slopes of the competitive equilibrium curves have the same significance as those of the non-competitive curves. We may therefore apply the same reasoning to the points through which curve C of Fig. 3 is drawn, and assign $n = 1$ to this reaction between enzyme and inhibitor in the presence of substrate. We should therefore conclude from these two experiments (unless there is some systematic error in the data) that *one molecule of acetylcholine or of physostigmine combines reversibly with a single molecule of cholinesterase.*

Eadie (5) studied the mechanism of combination of cholinesterase and physostigmine and concluded that in this reaction $n = 2$. We have just shown that on the basis of our studies on this same system such a mechanism is almost out of the question. But since we ruled out such a mechanism solely on the basis of slope, we shall now proceed to show that the observed effect of dilution is also incompatible with it. Straus and Goldstein showed that their experimental results on dilution of a dog serum-physostigmine mixture agreed quite well with the expected results calculated according to the dilution equation for $n = 1$. In Table I we have entered the experimental data of these authors (from their Table III) together with the values that would be expected for each concentration, if $n = 2$, according to Fig. 7. The discrepancies are not only excessively great, but follow an unmistakable trend rather than a random scatter, indicating the type of systematic error in calculated values which would result from use of an equation that did not correctly describe the reaction mechanism.

On the other hand there is no doubt that Eadie's conclusion follows from his data, which are in themselves consistent. Thus, if the data of his Fig. 4 are replotted according to our method, a curve with slope 1.15 is obtained, showing that $n = 2$ (in zone A). Therefore the problem of reconciling Eadie's results with our own comes down to showing, if possible, some systematic error in his data which could account for the discrepancy. His method involves the addition of enzyme to a substrate-inhibitor mixture followed by titration of released acetic acid against 0.01 N NaOH for 20 minutes. We have found that

under similar conditions the combination of physostigmine with enzyme is rather slow and that the hydrolytic rate in the first 20 minutes is significantly higher than at equilibrium, and this discrepancy is greatest for concentrations

TABLE I
Effect of Dilution on Activity, a , if $n = 2$

Serum concentration in per cent of undiluted serum	1.00	4.54	9.00	22.2	100
I/E					
1.1	0.980	0.725	0.470	0.175	0.015
	0.995	0.930	0.815	0.590	0.280
	0.995	0.965	0.900	0.745	0.490
	0.995	0.960	0.875	0.700	0.425
1.8		0.91		0.525	
		0.96		0.700	
2.1	0.98	0.725	0.47	0.175	0.015
	0.99	0.82	0.61	0.305	0.050
	0.99	0.88	0.72	0.445	0.125
	0.99	0.91	0.77	0.52	0.195
9.1		0.64		0.11	
		0.82		0.31	
10.7	0.81	0.125	0.025	0.005	~ 0
	0.92	0.46	0.20	0.04	~ 0
	0.955	0.59	0.32	0.08	0.005
	0.98	0.70	0.44	0.15	0.01
21.5	0.78	0.12	0.08	0.005	~ 0
	0.87	0.28	0.10	0.02	~ 0
	0.925	0.47	0.21	0.04	~ 0
	0.965	0.63	0.36	0.10	0.005
215	0.24	0.02	~ 0	~ 0	~ 0
	0.84	0.14	0.04	0.005	~ 0
	0.875	0.30	0.10	0.02	~ 0
	0.92	0.46	0.20	0.04	~ 0

of inhibitor producing moderate inhibitions. Since Eadie uses an equation for competitive equilibrium while such equilibrium (at least according to our experiments) does not really exist throughout the 20 minutes of his determination, the points of his Fig. 4 are warped downward precisely in the middle of his range of data, producing an apparent systematic divergence from linearity. The correspondence of the points to the curve for $n = 2$ would then be for-

tuitous. Our results, on the other hand, were obtained from readings in an *E-S-I* mixture at full equilibrium, and should not be subject to this error. However, it must be admitted that even the data of Straus and Goldstein, or our curve OBS of Fig. 3, which are obtained from readings made 3 to 23 minutes after substrate addition, agree with the mechanism $n = 1$ and are incompatible in a number of respects with $n = 2$.

One may wonder how far it is legitimate to apply these concepts to physiological phenomena. If a physiological effect is measured and a graded response obtained as a function of varying concentration of drug, can the slope of this function (when plotted as $da/d \log I$) be accorded the same significance as the slopes we have been discussing? This is difficult to decide if we are ignorant of the intermediary steps by which the effect is mediated and may not even know what enzyme is involved, or, for that matter, that enzyme inhibition is the responsible factor. Nevertheless it is interesting that one can demonstrate a variety of different types of reaction, both *in vitro* and *in vivo*, which give curves like those of Fig. 2 or Fig. 5 and whose slopes fall near the significant values 0.288, 0.575, or 1.151. Such similarities are usually obscured by the variety of methods used in plotting the data, but can be brought out by replotting according to the procedures we have outlined.

Such examples can be cited from the review of Clark (1), who not only presented such material in "dose-effect" curves of the type we have described, but also recognized the fact that the shape of these curves reflected the value of n . Thus the action of mercuric nitrate upon urease (data of Jacoby) is seen to be represented by a curve with slope 0.575 at the midpoint so that $n = 1$. The inhibition of blood catalase by silver nitrate (data of Bleyer) yields a slope 0.288 and therefore it would appear that $n = \frac{1}{2}$. Potassium cyanide acts upon peptidase (data of Linderstrøm-Lang) to give a curve with slope 1.151 so that $n = 2$.⁴

Interesting *in vivo* examples can be selected at random from the literature. Dubos and coworkers (8) found that a graded inhibition of the oxygen uptake of *S. aureus* cultures could be produced by varying concentrations of tyrocidine. When the data of their Table I (for 120 to 150 minutes after inhibitor addition) are plotted with a as ordinate (letting a equal per cent of normal $Q_0 \times 1/100$) and log concentration of tyrocidine as abscissa, a sigmoid curve is obtained whose slope is close to 0.575. This might be interpreted to mean that one molecule of tyrocidine combines reversibly with one molecule of the enzyme whose inhibition is the key factor in blocking respiration.

In a very different type of system Astwood and Bissell (9) have shown that varying concentration of thiouracil (which is presumed to inhibit an enzyme

⁴ Clark incorrectly concludes from this curve that $n = \frac{1}{2}$ so that one molecule of cyanide combines with two of peptidase, when actually the reverse is the case.

responsible for some phase of thyroxin synthesis) in the drinking water of rats produces a graded effect upon the iodine content of the thyroid. When the data of their Fig. 3 are plotted in the proper way (letting $a = 1$ when thyroid iodine is 77.5 mg. per cent, in the absence of inhibitor), a sigmoid curve of slope 0.575 is obtained. This may then indicate a 1:1 combination of thiouracil with the particular enzyme concerned. From the midpoint of this curve we may also obtain a value of K_I , which, although purely empirical, may still be quite useful. This constant is affected by such factors as the amount of inert protein binding inhibitor molecules, and the loss of inhibitor into tissues where it can have no effect. But as an empirical constant for given physiological conditions it still is a convenient comparative way of expressing inhibitor potency.

Of course in a complex reaction, the slope of the "dose-effect" curve may reflect only the mechanism of the *limiting stage* of the reaction, and not necessarily that of the crucial enzyme-inhibiting stage.

A word is in order here about the extent to which inhibition of an enzyme in serum reflects the inhibition of the same enzyme in tissues. It will be recalled from equation 2 that under all circumstances, in zones *A*, *B*, and *C*, the concentration of *free inhibitor*,

$$I_f = K_I \cdot \frac{1 - a}{a}$$

This states that the activity, a , of the enzyme is always determined by free inhibitor, for a given K_I . This seems at first to be contrary to the obvious fact that inhibition depends upon *combined* inhibitor. But if one imagines that a certain "pressure" of free inhibitor is always required to maintain a certain saturation of the enzyme, the contradiction will be resolved. If the inhibitor be a relatively small molecule, it will be clear that while E and EI are not diffusible, I_f should freely pass semipermeable membranes, so that *its* concentration ought to be the same within and without the vascular compartment, at equilibrium.⁵ And if free inhibitor in serum $\left(K_I \cdot \frac{1 - a_s}{a_s}\right)$ equals free inhibitor in tissues $\left(K_I \cdot \frac{1 - a_t}{a_t}\right)$, it follows that $a_s = a_t$. In other words, the enzyme inhibition is always the same in tissue as in serum, regardless of the zone and of the enzyme concentration in either, provided only that adequate time is left for diffusion, so that a true equilibrium is achieved. In the case of an enzyme which is present only in the tissues, measurement of the free

⁵ Davis (10) has verified this assumption for protein-bound and free sulfonamide equilibrated across a semipermeable membrane. The Donnan effect can probably be neglected.

inhibitor in the blood at equilibrium is sufficient for determining the degree of inhibition of the enzyme, for the same reasons. In either type of experiment diffusion as a significant factor can probably be overcome only with a continuous infusion technique; single doses of drug should produce a lag in inhibition in the tissues as compared with the circulating serum, and because of simultaneous excretion, destruction, etc., the expected level of inhibition may never be reached.

Several investigators (11, 12) have confirmed the fact that cholinesterase is not dialyzable, and that inhibition by physostigmine is reversible by dialysis. Preliminary experiments by the author appear to confirm the prediction that fractional activity of different concentrations of cholinesterase in the presence of physostigmine on two sides of a dialysis bag is the same when equilibrium is attained. Substitution of the red corpuscle for the dialysis bag with simultaneous determination of inhibition of serum and corpuscular cholinesterase would seem to be the next step along this line of experimentation.

We are fully aware of the hazards of attempting interpretation of the mechanism of reactions of whose very nature we are still ignorant. We wish, therefore, to emphasize the speculative character of the preceding discussions, whose conclusions lack as yet any direct experimental support.

Kinetic Studies

Experimental Method.—

The standard method employed for the experiments reported in this paper was as follows: Sterile dog serum, stored at 6°C. (there is no decrease in activity after months at this temperature) was used at a concentration of 4.54 per cent in the final reaction mixture. Substrate was 0.0805 M ($S' = 64.4$) acetylcholine bromide in the final reaction mixture, desiccator stored and freshly prepared for each experiment. Inhibitor was physostigmine salicylate prepared fresh to the desired concentration, which is always expressed as mols per liter reaction mixture. The method of determination was that of Ammon (13), using the Warburg constant volume manometer to measure release of CO_2 from a bicarbonate-Ringer solution medium buffered to pH 7.4. All determinations were made at 38°C. Readings were for the period 3 to 23 minutes after addition of 0.2 cc. substrate solution to 2.0 cc. containing enzyme with or without inhibitor. Appropriate correction for non-enzymatic hydrolysis was always made.

This method was altered as indicated in special experiments. Thus in the acetylcholine activity curve (Fig. 1 and Table II), the substrate concentration was varied as required. In curve C of Fig. 3 (Table III) the readings were 60 to 80 minutes after addition of an inhibitor-substrate mixture to the enzyme solution. In the experiments to follow, modifications of this standard technique will be described wherever they have been necessary.

Competitive Displacement of Inhibitor or Substrate.—

There are three ways to prove that inhibition is competitive. (1) The indirect method involves demonstrating in a plot of $1/v$ against $1/S$ that addition of inhibitor causes a change of slope but not of the intercept on the $1/v$ axis. This intercept is equal to $1/V_{\max}$, and while V_{\max} is reduced in non-competitive inhibition, it remains unchanged in the competitive type (Lineweaver and Burk (14)). (2) A more direct way of showing that inhibition is competitive is to prove that with substrate present a great deal more inhibitor is necessary to produce a given effect than in its absence. This we have already shown in Fig. 3, where the differences in inhibitor requirement between curve C (which is competitive) and curve OBS (which is nearly non-competitive) are quite striking in their magnitude. If one first produces half-inhibition in the determination made 3 to 23 minutes after adding substrate, then nearly 100 times as much inhibitor is required to reproduce this same inhibition in the 60 to 80 minute reading, when competitive equilibrium has been attained. (3) The most direct method of all, possible only with a technique that permits of continuous readings during the course of a reaction, is to follow the actual time course of entrance of substrate or inhibitor (as the case may be) into the reaction. The experiments about to be described are based on this principle.

In the first type of experiment (*A*, below) we study the entrance of inhibitor into an enzyme-substrate combination, with displacement of some of the combined substrate so that a shows a progressive fall. Serum and acetylcholine are first allowed to react for 30 minutes, during which¹ constant hydrolytic rate is attained, and then inhibitor, in varying concentration, is added. Readings are taken every 20 minutes until equilibrium is attained, so that the variation of a with time can be directly plotted.

In the second type of experiment (*B*, below) equilibrium between enzyme and inhibitor is first attained (incubation for 1 hour). Substrate is then added and readings taken at 3 minutes and every 20 minutes thereafter, to equilibrium. Here we follow the displacement of combined inhibitor by substrate and a consequently shows a progressive rise.

It was found in these experiments that a could not be calculated simply as the ratio of observed velocity to the *initial* velocity of uninhibited serum; nor could it be based upon the velocity of uninhibited serum at *equivalent time* as the reaction progressed. For under the conditions of the experiment there was a consistent decrease in the velocity of substrate hydrolysis in normal uninhibited serum with time. This decrease could even be observed between the first and second 20 minute periods, and became more marked as hydrolysis proceeded, until the reaction stopped entirely when about 1000 c. mm. CO_2 had been released (corresponding with exhaustion of the bicarbonate buffer). One could only correlate this decreasing velocity, barely perceptible though it was at the outset, with accumulation of hydrolytic products; whether the

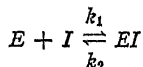
immediate cause was the increase in choline concentration or a shift in pH was immaterial. It was a fact that the maximal activity obtainable from the enzyme always depended upon the total CO_2 that had been released at the time. Therefore inhibited rates were always compared with the normal rate *at the same total hydrolysis*, the ratio of these two velocities being designated as a .

The time course of displacement will be shown to depend upon the absolute velocity constants for the combination of enzyme with inhibitor or substrate. The measurement of competitive displacement of one molecular species by another to determine a velocity constant, while unusual, is not without precedent. Francis and coworkers (15), in 1925, devised a similar experiment for determining the velocity constants of the rapid reactions between bromine and certain phenolic compounds. The uniqueness of the method lies in the fact that while the velocity of combination might be too great to measure directly, the presence of a reversibly combined complex enormously reduces the *immediately effective* concentration of the enzyme (E_f) without changing its ultimately effective total concentration. The resulting initial velocity of combination with the new reactant is so markedly diminished as to render it measurable by our relatively crude experimental methods.

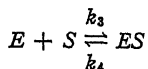
A. Displacement of S by I .—

Fig. 8 shows the progressive decrease in a after adding two different concentrations of inhibitor to an equilibrated enzyme-substrate system. Activity in each 20 minute period is plotted at the midpoint of the period. Since the points on a given curve represent consecutive readings in the same reaction mixture, it should not be unusual for a sizable error during one period to be compensated in the next (such a situation might arise from small absolute errors in the thermobarometer readings); the tendency of the points to seesaw is therefore not to be regarded as a serious experimental error.

The rate of displacement of S by I will depend upon the rate of combination of I with the enzyme, given by



and also by the rate of dissociation of the complex ES , given by



where k_1 , k_3 , and k_2 , k_4 are, respectively, the velocity constants for the forward and reverse reactions. Then,

$$\frac{d(ES)}{dt} = k_3(E_f)(S_f) - k_4(ES)$$

$$\frac{d(EI)}{dt} = k_1(E_f)(I_f) - k_2(EI)$$

Substituting for (E_f) in the first equation its value in the second, we obtain:

$$\frac{d(ES)}{dt} = \frac{k_3(S_f)}{k_1(I_f)} \left[\frac{d(EI)}{dt} + k_2(EI) \right] - k_4(ES)$$

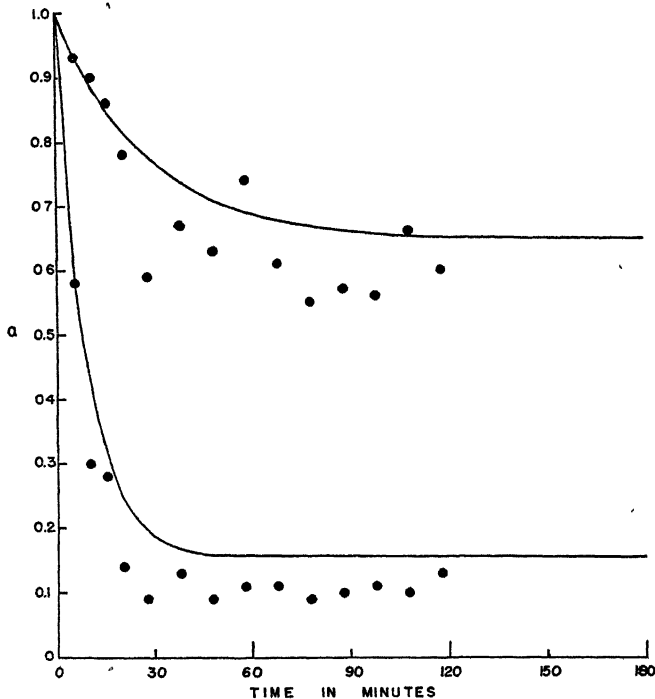


FIG. 8. Displacement of substrate by inhibitor. Physostigmine salicylate added at zero time to an equilibrated mixture of dog serum and acetylcholine. ● = observed activity for successive 20 minute periods with 1.1×10^{-6} and 1.1×10^{-5} M physostigmine, plotted at the midpoint of each period. Solid curves: theoretical course of the reaction, for these two concentrations of physostigmine, for $k_2 = 0.026$, and $k_4 = 0.32$.

We then assume (1) that we are in zone *A* with respect to substrate, so that practically $S_f \doteq S$, and (2) that free enzyme is negligible, so that $EI = (1 - a)E$; both these assumptions have been shown to be legitimate for the conditions of these experiments. Then, making the usual substitutions, with the above simplifications:⁶

$$\frac{da}{dt} = \frac{S'(1 - a) - (I_f)'a}{\frac{(I_f)'}{k_4} + \frac{S'}{k_2}} \quad (15)$$

⁶ It will be recalled that $k_2/k_1 = K_I$ and $k_4/k_3 = K_S$.

Knowing that $E < 1.8 \times 10^{-8}$ (page 544), we may neglect it by comparison with the lowest (I) used in this experiment (1.1×10^{-6} M), and we may assume that practically all I is free and write the zone A equation:

$$\frac{da}{dt} = \frac{S'(1-a) - I'a}{\frac{I'}{k_4} + \frac{S'}{k_2}} \quad (15A)$$

We can then substitute values of da/dt from the experimental curves at various values of a , to arrive by simultaneous solution at the values of k_2 and k_4 .

Integration of equation 15A and setting $a = 1$ when $t = 0$, gives

$$a = \frac{1}{S' + I'} \left\{ S' + I' e^{-\frac{(S' + I')t}{\frac{I'}{k_4} + \frac{S'}{k_2}}} \right\} \quad (16A)^\dagger$$

This equation can be used to construct theoretical curves giving a as a function of k_2 , k_4 , and t . The time scale can then be adjusted to give the best fit to the experimental points, this choice of scale automatically giving the best values of the velocity constants.

It is of interest that if the rigid equation 15 be rewritten for $a = 1$, to describe the *initial* rate of change of a , this change is seen to be entirely independent of the enzyme concentration; since at the start of the reaction all inhibitor is free and no assumptions are necessary:

$$\left(\frac{da}{dt} \right)_{a=1} = - \frac{I'a}{\frac{I'}{k_4} + \frac{S'}{k_2}}$$

Using any of these three methods, we find that $k_2 = 0.026$ minutes⁻¹ so that $k_1 = k_2/K_I = 8.3 \times 10^5$ liters·mols⁻¹·minutes⁻¹; and $k_4 = 0.32$ minutes⁻¹ so that $k_3 = k_4/K_S = 260$ liters·mols⁻¹·minutes⁻¹.

The solid lines of Fig. 8 are the theoretical curves drawn according to equation 16A, with the above velocity constants; their fit to the experimental points is considered to be satisfactory.

B. Displacement of I by S .—

The points of Fig. 9 show the progressive increase in a after substrate is added to an equilibrated mixture of E and I . The validity of the equilibrium competitive curve (C, Fig. 3) is strengthened by the fact that the same equilibrium is attained with a given I and S , regardless of the order of addition of reactants. The points on which that curve is based were obtained by preliminary equilibration of E , S , and I . The two black squares in Fig. 3 represent the ultimate equilibria attained by the curves of Fig. 8; the five white squares represent the

[†] As $t \rightarrow \infty$, $a \rightarrow \frac{S'}{I' + S'}$, which is the zone A value of a at competitive equilibrium.

equilibria attained by the curves of Fig. 9. It was also found that in the experiments of Fig. 9, the same equilibrium was approached if substrate was added an hour after the usual time; this was taken to mean that the presence

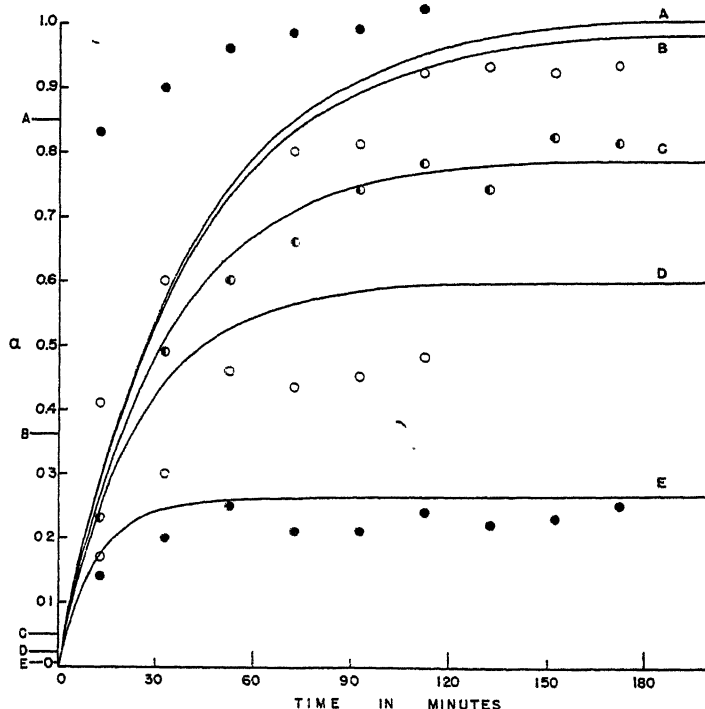


FIG. 9. Displacement of inhibitor by substrate. Acetylcholine added at zero time to an equilibrated mixture of dog serum and physostigmine. ● and ○ = observed activities for successive 20 minute periods, for each of the five physostigmine concentrations represented by the solid curves A to D, plotted at the midpoint of each period. Solid curves: theoretical course of the reaction, for $k_2 = 0.026$ and for $k_4 = 0.32$, for each of the following concentrations of physostigmine: A— 5.5×10^{-9} M; B— 5.5×10^{-8} M; C— 5.5×10^{-7} M; D— 1.35×10^{-8} M; E— 5.5×10^{-8} M. On the ordinal axis are indicated by the appropriate letters the value of the E_f/E ratio for each physostigmine concentration before addition of substrate.

of inhibitor for a longer time did not in itself cause any change in the potential activity of the enzyme. The increase in reaction rate during the second 20 minute period as compared with the first when an inhibitor is present is in marked contradistinction to the normal slight decrease in rate of the uninhibited enzyme. This phenomenon is so reliable that it can be used as a criterion for the presence of a reversible inhibitor in an unknown solution of enzyme.

The displacement of combined inhibitor by added substrate is represented by the same equations already derived for the displacement of substrate by inhibitor, namely equations 15 and 15A, which describe the rate of change of a in terms of both velocity constants, without reference to the order of addition of reactants. For the initial rate, a is set equal to zero in equation 15A, yielding, for zone A:

$$\left(\frac{da}{dt}\right)_{a=0} = \frac{S'}{\frac{I'}{k_4} + \frac{S'}{k_2}}$$

To derive the theoretical curves describing the course of the reaction, equation 15A must be integrated after setting $a = 0$ when $t = 0$, giving

$$a = \frac{1}{S' + I'} \left\{ S' - S' e^{-\frac{(S' + I')t}{\frac{I'}{k_4} + \frac{S'}{k_2}}} \right\} \quad (17A)$$

The solid lines of Fig. 9 are the theoretical curves for five concentrations of inhibitor, plotted according to this equation, with the velocity constants previously derived. While the fit of these curves to the experimental points is not remarkably good, we believe that the deviation from the lower four curves can be explained on the basis of experimental error; our analytic method has, after all, presupposed data more accurate than our relatively crude technique could furnish. While the lower curves are consistent with the observed course of the reaction, this is certainly not true for curve A, representing the smallest inhibitor concentration. We are unable to explain this discrepancy except to point out that it does *not* arise from the assumption of zone A in a system which may really be in zone B. For while the use of I' instead of $(I_f)'$ may result in a false high value for this term, it is already (at this inhibitor concentration) so small as to be negligible in the wholly additive relationships in which it is found (*cf.* equation 17A); thus large differences in the value of I' at this concentration are without effect on the equation as a whole.

Whether the experimental points for curve A are at fault, or whether (as seems more likely) our treatment does not correctly describe the reaction at low inhibitor concentrations, the practical consequence of this discrepancy is equally unfortunate. For we should like to be able, on a theoretical basis, to correct any 3 to 23 minute reading to its true competitive value, from which the non-competitive EI/E ratio could be calculated. Without an acceptable equation which will fit the whole range of data, we must limit ourselves to empirical corrections. (For a full discussion of this and other corrections which must be applied to experimental observations, see Kraye, Goldstein, and Plachte (16), Discussion.)

On the ordinal axis of Fig. 9 we have indicated for each curve the non-com-

petitive value of E_f/E before addition of substrate. It might be thought that each curve would begin at that point on the a axis corresponding to its E_f fraction at zero time. This is not actually the case, for at zero time no ES is present and a must therefore be zero regardless of the amount of E_f or EI in the system. For this reason, which is well illustrated by the curves, extrapolation of the experimental points back to zero time is not a valid method of determining the initial non-competitive inhibition.

Reactions Depending on the Velocity Constants.—

The course of every conceivable reaction between two reactants at a given temperature is determined by their characteristic velocity constants. Thus, knowing k_1 and k_2 for the cholinesterase-physostigmine system, we are in a position to predict the time course of both the dilution effect and the simple combination which occurs when one reactant is added to the other.

A. Kinetics of the Dilution Effect.—

Straus and Goldstein's demonstration of the dissociation of an enzyme-inhibitor complex on dilution was based upon equilibrium considerations. We are now in a position to investigate the rate at which this occurs. If it is infinitely slow, it is of no practical consequence to the experimenter. If it is moderately slow, and determinations are made upon diluted serum before dissociation is complete, neither the uncorrected nor the fully corrected (for dilution) data will be accurate. Dilution corrections can only be applied *in toto* if the dissociation is very rapid, or if the diluted serum is allowed to stand long enough before determination for equilibrium to be achieved.

Let us again write the equation for change of (EI) with time:

$$\frac{d(E')}{dt} = k_1(E_f)(I_f) - k_2(E')$$

Letting $E_f/E \equiv a$ (since S is absent),

$$I_f \doteq I \text{ (zone A)}$$

$$EI = (1 - a)E$$

then,

$$\frac{da}{dt} = -k_1[(I + K_I)a - K_I] \quad (19A)$$

Now let I before dilution be I_0 , and let I after dilution be NI_0 , where N is the factor of dilution. Integrating equation 19A, making the above substitutions, and setting $I = I_0$ when $t = 0$, we arrive at:

$$a = \frac{K_I}{NI_0 + K_I} \left\{ 1 + \left(\frac{NI_0 + K_I}{I_0 + K_I} - 1 \right) e^{-k_1 t (NI_0 + K_I)} \right\} \quad (20)$$

We may eliminate I_0 entirely by substituting its original non-competitive value

$$I_0 = \frac{K_I(1 - a_0)}{a_0}$$

to get an equation relating a at time t to a_0 before dilution.

$$a = \frac{a_0}{N - (N - 1)a_0} \left\{ 1 + [N - (N - 1)a_0 - 1]e^{-\frac{k_2 t [N - (N - 1)a_0]}{a_0}} \right\} \quad (21)$$

From equation 21 one can plot theoretical dilution dissociation curves in zone A for any dilution factor N and any initial activity a_0 . In Fig. 10 we have done this for two initial activities in serum, diluted to 4.54 per cent and 9.09 per cent, using the value of k_2 obtained previously. The two pairs of solid curves in the lower part of the graph show this and the points represent data from an experiment designed to confirm this slow effect of dilution. In the experiment, serum containing inhibitor was diluted to the desired concentration at zero time, placed at 38°C. within 20 minutes, incubated for 20 minutes, and then determined against substrate which was introduced at various times. The points given are the observed activities; they are undoubtedly too high because they are 3 to 23 minute readings and therefore subject to the competition error already discussed; and there should also be a small correction for destruction of physostigmine during the experiment. The fit is therefore not too satisfactory, but the general magnitude of the time course certainly agrees with that predicted by the curves.

The pair of curves in the upper part of the graph is purely theoretical and is illustrative of the course of events on dilution to 22.2 per cent and 1.0 per cent of serum with 56.3 per cent of normal activity initially (one-tenth the inhibitor concentration used in the upper of the lower pairs of curves).

The time course of dissociation on dilution is seen to be moderately slow. The dilution correction must be applied to experimental results with this fact in mind, and experiments should preferably be designed with an equilibration period *after* dilution long enough for dissociation to be completed.⁸

B. Kinetics of Combination.—

A number of investigators have observed that the attainment of equilibrium between physostigmine and cholinesterase is a slow process, and various expedients, such as storage overnight in the ice chest, have been adopted to insure

⁸ The solution of the dilution-time problem for zone B follows the same steps as above but nothing would be gained by presenting the equation (which involves an integral of the form $\int \frac{da}{Ca^2 + Ba + A}$).

complete equilibration before any determinations are made. No quantitative work has come to our attention describing *how* slowly the combination proceeds, nor how great an error is involved in determining activity at some time before equilibrium.

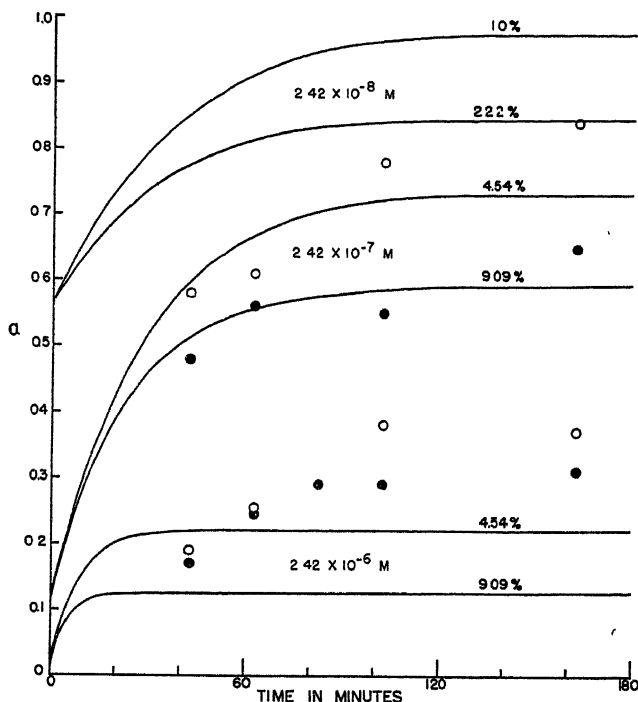


FIG. 10. Kinetics of the dilution effect. The two lower pairs of curves represent the theoretical time course of the dilution effect, for $k_2 = 0.026$, when sera containing 2.42×10^{-7} M and 2.42×10^{-6} M physostigmine are diluted at zero time to 4.54 per cent and 9.09 per cent. ● = observed activities (plotted at the midpoints of the 20 minutes periods) for 9.09 per cent. ○ = observed activities for 4.54 per cent. These points are not corrected for destruction or competition, so that all values of a are too high. The upper pair of curves shows the theoretical course of the reaction when serum containing 2.42×10^{-8} M physostigmine is diluted to 22.2 per cent and to 1.0 per cent.

Combination is obviously determined by the velocity constants k_1 and k_2 . This combination rate is given by equation 19A, which may be integrated directly, setting $a = 1$ when $t = 0$, giving:

$$a = \frac{1}{I' + 1} \{1 + I'e^{-k_2 t(I' + 1)}\} \quad (22)$$

Using the value of k_2 already obtained we have plotted, in Fig. 11, the theoretical curves showing the course of combination of added inhibitor with enzyme in zone *A*. This time course is in substantial agreement with our own experience and with the results which have appeared in the literature. The curves show, as would be expected, that combination is almost immediate with high concentrations of *I* and very slow with low concentrations. The error

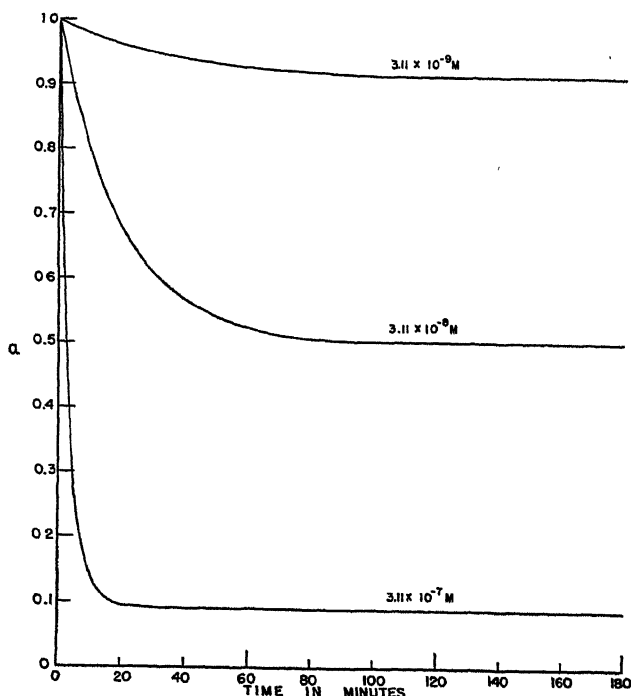


FIG. 11. Combination of inhibitor with enzyme. The theoretical time course of the reaction, for $k_2 = 0.026$, when three concentrations of physostigmine are added to dog serum (zone *A*).

in determining a at, let us say, one-half hour would be greatest (about 0.07) for medium values of *I*.

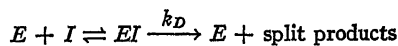
The time course of combination is independent of *E* in zone *A*. For zone *B*, it can be obtained by integrating, with respect to a , the equation:

$$\frac{da}{dt} = -k_2[E'a^2 + (I' - E' + 1)a - 1] \quad (19B)$$

Destruction of Inhibitor or Substrate.—

We shall consider the enzymatic destruction of inhibitor, bearing in mind that we are thereby treating it as a substrate, and that consequently the same

considerations will apply to substrate breakdown itself. Inhibitor destruction may be described as follows:



where k_D is the velocity constant of destruction. Then,

$$\frac{dI}{dt} = -k_D(EI) \quad (23)$$

Substituting,

$$I = K_I \frac{(1-a)}{a} + (1-a)E$$

$EI = (1-a)E$, and the other substitutions as usual:

$$\frac{da}{dt} = \frac{k_D(1-a)}{\frac{1}{E_I' a^2} + 1} \quad (24)$$

Integrating, and setting $a = a_0$ when $t = 0$:

$$\left[\frac{1}{a_0} + \ln \frac{1-a_0}{a_0} - E_I' \ln(1-a_0) \right] - \left[\frac{1}{a} + \ln \frac{1-a}{a} - E_I' \ln(1-a) \right] = k_D E_I' t \quad (25)$$

In this equation the terms on the left containing E_I' are insignificant even when E_I' is considerably greater than 0.1, so it is safe to reduce it to the zone *A* form:

$$\left[\frac{1}{a_0} + \ln \frac{1-a_0}{a_0} \right] - \left[\frac{1}{a} + \ln \frac{1-a}{a} \right] = k_D E_I' t \quad (25A)$$

Equation 25A allows one to plot the theoretical curve of destruction starting with any initial activity a_0 , as a function of $k_D E_I' t$. We then require an experimental curve to which we can adjust the time scale, a procedure which at the same time gives the best fit of the data to the theoretical curve and also the best value for the term $k_D E_I'$. There is no way of solving for either of these constants separately by means of the destruction function alone. If E_I' can be determined by other means, we will then know k_D , but in the series of experiments described in this paper we have shown that it is impossible to settle on any but a maximum value of E_I' (< 0.58 for cholinesterase in 4.54 per cent dog serum).

Ellis, Plachte, and Straus (17) made a study of the course of physostigmine destruction in horse serum and plotted the increase of a with time in their Fig. 4. Accepting their experimental data and fitting our theoretical curve (be-

ginning with $a_0 = 0.08$) to their "corrected serum curve," we obtain curve B of Fig. 12, which is an almost identical fit to their data. When such a fit is made, the value obtained for $k_D E_I'$ is 1.4. Using this value we have plotted

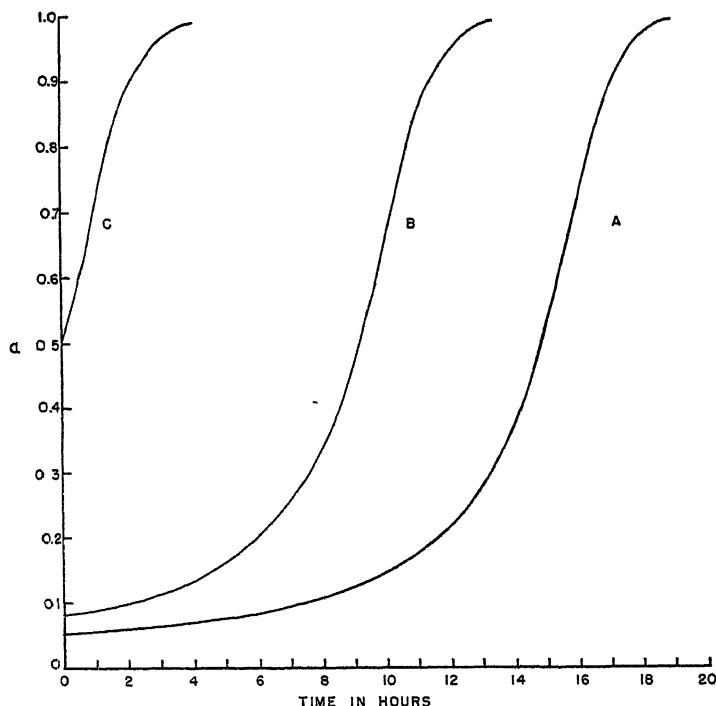


FIG. 12. Enzymatic destruction of inhibitor or substrate. The theoretical time course of the increase in fractional activity is shown for different initial activities. The time scale is so chosen that the experimental data of Ellis, Straus, and Plachte for cholinesterase and physostigmine will be fitted by curve B. The resulting destruction constant, $k_D = 0.00182 \text{ minutes}^{-1}$, is used to plot curves A and C. All possible destruction curves with initial activity greater than 0.05 are really segments of curve A, which is the major part of the single curve that represents the course of this reaction. These curves could be generalized by using $k_D E_I' t$ as abscissa, and made valid for substrate by then substituting $(1 - a)$ for a on the ordinal axis (see text).

similar curves (A, C of Fig. 12) beginning at different initial activities. These, however, are actually all segments of a *single* destruction curve, represented in its major part by curve A. That all the other curves should be identical with some segment of curve A arises from the fact that the destruction rate is proportional to the activity a at any time, regardless of the previous history of the reaction.

This destruction curve, which applies to the combination of a single molecule

of inhibitor with a molecule of enzyme, is characterized by an infinitely slow increase in a when a is practically zero (although *absolute rate of inhibitor destruction* is most rapid here). The rate of change of a slowly increases, becoming quite rapid at about 0.20. The most rapid change occurs at 0.667,⁹ where the curve inflects; and very close to maximal activity the slope falls off quite rapidly, the rate of change of a becoming again infinitely slow as a approaches 1.

For the change in a when substrate is being destroyed, the reasoning is identical, giving the same curve, except that $(1 - a)$ must be substituted for a in all the equations and on the ordinate of the destruction-time curve, the time scale being determined by k_D for the given substrate.

Ellis, Plachte, and Straus studied the spontaneous as well as the enzymatic breakdown of physostigmine, and deriving the equation

$$k_D = \frac{2.3}{at} \log \frac{b}{b - x} \quad (26)$$

plotted $\log \frac{b}{b - x}$ against time to obtain an estimate of k_{Da} . Here b represents initial physostigmine, x the amount destroyed, and a the concentration of the other reactant—either enzyme or hydroxyl ion. They found that while the expected linear plot was obtained for the hydrolytic breakdown, the enzymatic reaction yielded a linear curve only after 6 hours. Their conclusion was that both this phenomenon and the observed slow rate of change of a at the beginning of the destruction-time curve represented an "inhibition due to excess substrate (physostigmine)."

But we have already demonstrated that the initial slow change in a is perfectly typical of the expected reaction mechanism and that there is no need to assume inhibition by excess substrate. The incorrect conclusion arrived at by these authors can be shown to arise from an invalid approximation in the derivation of their equation for enzymatic destruction of inhibitor.

Equation 26 is derived from the relation:

$$dx/dt = k_D ba \quad (27)$$

In our terminology this amounts to:

$$-dI/dt = k_D(\text{OH}^-)I, \quad \text{for hydrolysis} \quad (28)$$

and

$$-dI/dt = k_D \cdot E \cdot I, \quad \text{for enzymatic breakdown.} \quad (29)$$

Let us first take up equation 29, in which the rate is said to depend upon the product of inhibitor by *total* enzyme concentration—the common "mono-molecular assumption." We know that the rate in fact depends upon *com-*

⁹ This is arrived at by setting $d^2a/dt^2 = 0$ in equation 24.

bined enzyme concentration, according to equation 23. These two equations are only truly *equivalent* when $E \cdot I = (EI)$ and this is never the case for any real values of a . However, they *are* of the same *form* if a linear function relates (EI) to E and I , so that

$(EI) = c \cdot E \cdot I$ for then equation 29 becomes

$$-dI/dt = \frac{k_D}{c} (EI) = k_D' (EI)$$

Thus it is legitimate to assume a monomolecular reaction mechanism for the destruction of inhibitor (or substrate) by enzyme only if (1) there is no intermediate and the reaction is irreversible; or (2) a reversibly formed intermediate breaks down so rapidly that its existence may be neglected; or (3) an intermediate, whose concentration may be appreciable, is formed by the stoichiometric combination of the initial reactants. The monomolecular reaction mechanism does not apply under other circumstances which are frequently encountered. Specifically, in the case under discussion, the concentration of the intermediate (EI) is appreciable and its reversible formation from E and I is not stoichiometric; consequently, the destruction rate cannot be assumed to depend upon the concentrations of the initial reactants, but only upon that of (EI) .¹⁰

The effect of this fallacy in the theoretical premises of the above cited authors may be illustrated by repeating for the correct equation 23 the steps of substitution and integration which were used to derive their equation 26 from the incorrect equation 29, yielding:

$$k_D = \frac{2.3K_I}{at} \left[\log \frac{b}{b-x} + \frac{x}{2.3K_I} \right] \quad (30)$$

If $\log \frac{b}{b-x}$ is now plotted against time we shall obviously have a curve which is not linear but deviates progressively from linearity—as a matter of fact this equation now perfectly describes the experimental points of Ellis, Plachte, and Straus' Fig. 4. In other words both their predication of a linear plot and their assumption of an unusual reaction mechanism (inhibition by excess inhibitor) to explain the observed non-linearity are unwarranted, the reaction actually following the expected theoretical course without deviation.

As we should expect from the foregoing discussion, the value of k_D obtained by these authors (2.74×10^6) has no relation to the correct constant, for not only its magnitude but its dimensions are different. For our maximum value

¹⁰ The reader is referred here to Northrop's (18) discussion of this question and his conclusion that in the trypsin-gelatin-trypsin inhibitor system an enzyme-inhibitor but not an enzyme-substrate complex is formed.

of E_I' (12.8 in whole serum), $k_D = 0.00182$. Since E_I' may be much smaller, as we have shown, this must be regarded as the minimum value of k_D .

It is interesting in view of the discrepancies arising from the use of equation 29 that equation 28, which resembles it superficially, should give such satisfactory results (*cf.* Figs. 3A and 3B of the above authors). The reason for this is that the two equations are not really analogous, for the term (OH^-) is not *total* but *free* hydroxyl.¹¹ Because this equation is stated only in terms of a *free* reactant, no false assumptions are involved and it is perfectly valid. Equation 29 would be equally correct if written in terms of *free* rather than *total* enzyme;¹²

$$-\frac{dI}{dt} = k_D \cdot E_I \cdot I = k_D(aE) \left(\frac{1-a}{a} \right) = k_D(1-a)E = k_D(EI)$$

and it has become identical with the legitimate zone A equation 23.

SUMMARY

The mechanism of enzyme-inhibitor-substrate reactions has been analyzed from a theoretical standpoint and illustrated by data from the system cholinesterase-physostigmine-acetylcholine. This treatment is by no means limited to a single system but should be generally applicable to others of similar type.

Competitive enzyme-inhibitor-substrate systems show the same characteristic "zones of behavior" already demonstrated for non-competitive systems by Straus and Goldstein. These zones, three in number, determine the mathematical function which relates activity of an enzyme to concentration of an added substrate or inhibitor or both.

The effects of suboptimal substrate concentration in such systems have been considered, and the errors arising from various common simplifications of the descriptive equations have been pointed out.

The zone behavior phenomenon has been shown to be useful in determining the number of molecules of substrate or inhibitor combining reversibly with a single enzyme center.

The kinetics of competitive inhibition, dilution effect, combination of inhibitor or substrate with enzyme, and destruction of inhibitor or substrate by enzyme have been analyzed and experimentally verified, and absolute velocity constants have been determined.

Theoretical conclusions have been discussed from the standpoint of their physiological significance.

¹¹ We determine the *free* (OH^-) but do not measure the *total* hydroxyl, including that which has combined with I .

¹² This recalls the footnote discussion in Straus and Goldstein, page 568, on the Henderson-Hasselbalch equation.

Specifically, it has been shown that:

1. The inhibition of cholinesterase by physostigmine is competitive. A single molecule of physostigmine or acetylcholine combines with one center of cholinesterase— $n = 1$; and the mechanism $n = 2$ has been excluded. Numerical values of the constants for this system are as follows:¹³

$$\begin{aligned} K_I &= 3.11 \times 10^{-8} \\ k_1 \text{ (combination)} &= 8.3 \times 10^5 \\ k_2 \text{ (dissociation)} &= 0.026 \\ K_S &= 1.25 \times 10^{-8} \\ k_3 \text{ (combination)} &= 260 \\ k_4 \text{ (dissociation)} &= 0.32 \end{aligned}$$

2. No definitive value can be assigned to E , the molar concentration of enzyme centers, but in 4.54 per cent dog serum, $E < 1.8 \times 10^{-8}$ ($E_I' < 0.58$). The system therefore operates in (or nearly in) zone *A* at this concentration.

3. Competitive displacement of inhibitor by substrate and *vice versa* introduces considerable error in the usual 20 minute determination of the activity of an inhibited enzyme, unless properly corrected for.

4. Dissociation of the enzyme-inhibitor complex on dilution proceeds moderately slowly so that the full corrections for dilution cannot be applied unless time has been allowed for full dissociation.

5. Combination of physostigmine with cholinesterase is slow at all but large concentrations of inhibitor.

6. The destruction of physostigmine or acetylcholine by cholinesterase follows the predicted curve; k_D for the destruction of physostigmine is found to be > 0.00182 ; k_D for acetylcholine destruction is > 3500 .¹³ There is no reason to assume inhibition of destruction by excess substrate or inhibitor.

7. The common assumption that enzymatic activity follows (or nearly follows) a monomolecular course is true only under limited conditions, which have been here defined. It is not valid, as a rule, for the enzymatic destruction of an inhibitor (*e.g.*, physostigmine) and its application to such a case may lead to erroneous conclusions about the reaction mechanism.

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¹³ All constants are expressed in terms of mols, liters, and minutes.

APPENDIX

TABLE II

Acetylcholine Activity Curve

(Data for Fig. 1)

Concentration of acetylcholine bromide (mols per liter reaction mixture)	Average observed velocity (c.mm. CO ₂ per 20 min.)	
2.02×10^{-1}	65.7	(1)*
1.61×10^{-1}	64.5	(5)
8.06×10^{-2}	63.1	(2)
5.04×10^{-2}	61.0	(1)
4.04×10^{-2}	61.3	(3)
2.02×10^{-2}	58.1	(2)
1.26×10^{-2}	51.5	(1)
1.01×10^{-2}	55.0	(3)
5.04×10^{-3}	49.8	(2)
3.16×10^{-3}	43.8	(1)
2.54×10^{-3}	47.3	(1)
1.26×10^{-3}	33.7	(2)
7.90×10^{-4}	25.5	(1)
6.34×10^{-4}	24.9	(1)
3.16×10^{-4}	11.0	(2)
1.97×10^{-4}	7.1	(2)
1.58×10^{-4}	8.3	(2)
Non-enzymatic hydrolysis		
1.0×10^{-2}	0.25	(1)
1.26×10^{-2}	0.75	(1)
4.0×10^{-2}	3.50	(1)
5.0×10^{-2}	3.50	(1)
1.62×10^{-1}	20.0	(1)
2.0×10^{-1}	21.5	(1)

* Numbers in parentheses indicate the number of determinations included in the average.

TABLE III
(Data for Fig. 3)

Curve C	
Concentration of physostigmine salicylate (mols per liter reaction mixture)	α
8.7×10^{-8}	0.95*
3.8×10^{-7}	0.85
7.8×10^{-7}	0.75*
8.3×10^{-7}	0.73*
1.7×10^{-6}	0.52*
3.1×10^{-6}	0.39
3.3×10^{-6}	0.36*
4.4×10^{-6}	0.33
6.6×10^{-6}	0.22*
8.7×10^{-6}	0.18
1.3×10^{-5}	0.10*
2.4×10^{-5}	0.08
Curve OBS	
Concentration of physostigmine salicylate (mols per liter reaction mixture)	α (based on velocities 3 to 23 min. after S added)
1.1×10^{-9}	~ 1.00
2.2×10^{-9}	0.94*
4.4×10^{-9}	0.90
5.5×10^{-9}	0.83*
6.05×10^{-9}	0.90
1.1×10^{-8}	0.83
1.1×10^{-8}	0.80
2.2×10^{-8}	0.66*
2.76×10^{-8}	0.63
5.5×10^{-8}	0.41*
5.5×10^{-8}	0.44
5.5×10^{-8}	0.46
6.05×10^{-8}	0.48
1.1×10^{-7}	0.33
1.1×10^{-7}	0.33
2.2×10^{-7}	0.25*
5.5×10^{-7}	0.23
5.5×10^{-7}	0.23*
1.35×10^{-6}	0.17*
5.5×10^{-6}	0.18*
5.5×10^{-6}	0.12
6.05×10^{-6}	0.14

* Indicates average of satisfactory duplicate determinations.

TABLE IV
Displacement of *S* by *I*
(Data for Fig. 8)

(Min. after <i>I</i> added)	<i>I</i> (mols per liter reaction mixture)	
	1.1×10^{-6}	1.1×10^{-5}
<i>t</i>	<i>a</i>	<i>a</i>
5.5	0.93	0.58
10.5	0.90	0.30
15.5	0.86	0.28
20.5	0.78	0.14
28	0.59	0.09
38	0.67	0.13
48	0.63	0.09
58	0.74	0.11
68	0.61	0.11
78	0.55	0.09
88	0.57	0.10
98	0.56	0.11
108	0.66	0.10
118	0.60	0.13

TABLE V
Displacement of *I* by *S*
(Data for Fig. 9)

(Min. after <i>S</i> added)	<i>I</i> (mols per liter reaction mixture)				
	5.5×10^{-6}	1.35×10^{-6}	5.5×10^{-7}	5.5×10^{-8}	5.5×10^{-9}
<i>t</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
13	0.14	0.17	0.23	0.41	0.83
33	0.20	0.30	0.49	0.60	0.90
53	0.25	0.46	0.60	0.73	0.96
73	0.21	0.43	0.66	0.80	0.98
93	0.21	0.45	0.74	0.81	0.99
113	0.24	0.48	0.78	0.92	1.02
133	0.22	—	0.74	0.93	—
153	0.23	—	0.82	0.92	—
173	0.25	—	0.81	0.93	—
223	—	0.50	—	—	—
243	—	0.54	—	—	—

TABLE VI
Time Course of Dilution
 (Data for Fig. 10)

Observed activities (3 to 23 minutes after *S* addition), uncorrected for competition or destruction.

(Min. after dilution)	$I = 2.42 \times 10^{-7}$ mols per liter serum	
<i>t</i>	9.09 per cent	4.54 per cent
43	0.48	0.58
63	0.56	0.61
103	0.55	0.78
163	0.65	0.84
283	0.68	0.97
	$I = 2.42 \times 10^{-6}$ mols per liter serum	
43	0.17	0.19
63	0.25	0.25
83	—	0.29
103	0.29	0.38
163	0.31	0.37
253	—	0.42
283	0.24	—

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